WALTER AND ANDRÉE DE NOTTBECK FOUNDATION
SCIENTIFIC REPORTS
No. 47

ECOLOGICAL AND EVOLUTIONARY ROLE OF SEED BANKS FOR THE TOXIC DINOFLAGELLATE *ALEXANDRIUM OSTENFELDII*

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Academic dissertation to be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki, in room 2041, Biocenter 2, Viikinkaari 5 and online via Zoom on 12th of November 2020 at 10 am.

Helsinki 2020

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ISBN 978-951-51-6716-3 (paperback)
ISBN 978-951-51-6717-0 (PDF, http://ethesis.helsinki.fi)
Helsinki 2020
Unigrafia Oy

This thesis is based on the following papers, which are referred to by their Roman numerals:

I. **Jerney, J.,** Rengefors, K., Nagai, S., Krock, B., Sjöqvist, C., Suikkanen, S., Kremp. A. (unpubl.): Seasonal genotype dynamics of a Baltic dinoflagellate – pelagic populations are homogeneous and as diverse as benthic seed banks*

II. **Jerney, J.**, Ahonen, S.A., Hakanen, P., Suikkanen, S., Kremp, A., 2019. Generalist life cycle aids persistence of *Alexandrium ostenfeldii* (Dinophyceae) in seasonal coastal habitats of the Baltic Sea. J. Phycol. 55, 1226–1238. https://doi.org/10.1111/jpy.12919.**

III. **Jerney, J.**, Suikkanen, S., Lindehoff, E., Kremp, A., 2019. Future temperature and salinity do not exert selection pressure on cyst germination of a toxic phytoplankton species. Ecol. Evol. 9, 4443–4451. https://doi.org/10.1002/ece3.5009.**

IV. Sildever, S., **Jerney, J.**, Kremp, A., Oikawa, H., Sakamoto, S., Yamaguchi, M., Baba, K., Mori, A., Fukui, T., Nonomura, T., Shinada, A., Kuroda, H., Kanno, N., Mackenzie, L., Anderson, D.M., Nagai, S., 2019. Genetic relatedness of a new Japanese isolates of *Alexandrium ostenfeldii* bloom population with global isolates. Harmful Algae 84, 64–74.***

* The manuscript will be submitted to Molecular Ecology

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CONTRIBUTION OF THE AUTHORS

	I	**II**	**III**	**IV**
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Sampling	**JJ,** AK, SS	**JJ**, PH	**JJ**	MY
Provision of isolates	**JJ**, AK	**JJ**	**JJ**	**JJ**, AK, AM, DA, HO, KB, LM, SSa
Data generation	**JJ**, AK, BK, KR, SN	**JJ**, AK, PH, SAA, SS	**JJ**, AK, EL, SS	SSi, **JJ,** SN
Data analysis	**JJ,** CS	**JJ**	**JJ**	SSi, AS, NK, SN
Manuscript writing	**JJ**	**JJ**	**JJ,** AK	SSi
Provided data / information				HO, AM, HK, KB, TF, TN
Contribution to the manuscript	AK, BK, CS, KR, SN, SS	AK, PH, SAA, SS	EL, SS	**JJ**, AK, AM, AS, DA, HK, HO, KB, LM, MY, NK, SSa, TF, TN

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ABBREVIATIONS

AMOVA – analysis of molecular variance
ANOVA – analysis of variance
BIC - bayesian information criterion
C – control treatment
CTAB – cetyltrimethylammonium
DAPC – discriminant analysis of principal components
GYM – gymnodimines
HABs – harmful algal blooms
I_A – index of association
MS – microsatellite markers
PST – paralytic shellfish toxins
PCR – polymerase chain reaction
PSU – practical salinity units
PCA – principal components analysis
RAD – restriction site associated DNA sequencing
$\bar{r}d$ – index of association adjusted for the number of loci
ITS – ribosomal DNA region: internal transcribed spacer
LSU – ribosomal DNA region: large subunit
S – salinity treatment
SSU – ribosomal DNA region: small subunit
SNP – single nucleotide polymorphisms
SPX – spirolides
T – temperature treatment
TS – combined temperature and salinity treatment

Ecological and evolutionary role of seed banks for the toxic dinoflagellate *Alexandrium ostenfeldii*

Jacqueline Jerney

Jerney, J. 2020: Ecological and evolutionary role of seed banks for the toxic dinoflagellate *Alexandrium ostenfeldii*. W. and A. de Nottbeck Foundation Sci. Rep. 47: 1–41. ISBN 978-951-51-6716-3 (paperback); ISBN 978-951-51-6717-0 (PDF)

ABSTRACT

Phytoplankton plays a pivotal role for aquatic ecosystem functioning and global biogeochemistry. Climate change has affected phytoplankton community composition and distribution in the last decades, including a higher prevalence for harmful algal blooms in many areas. The globally distributed dinoflagellate *Alexandrium ostenfeldii* has for example started to form dense toxic blooms in the Baltic Sea and a new bloom location was recently discovered in western Japan. To survive unfavorable conditions this species forms resting stages, which may accumulate in sediments, forming a "seed bank". The aim of this thesis was to investigate the relevance of the seed bank for the ecology and evolution of *A. ostenfeldii* and to understand the implications of these findings for persistence and possible expansion under ongoing global change.

A combination of field surveys in Finland and Japan, experimental work and genotyping were carried out to address these aims. The results indicate that the seed bank stores a large clonal diversity, underlining its importance for stabilizing local populations against environmental fluctuations. No population structure was detected in temporal parts of a pelagic population, showing that differentiation does not happen during one season. The life cycle of *A. ostenfeldii* was found to be highly versatile, allowing overwintering of asexual resting stages without a pronounced dormancy period, and sexual reproduction throughout the season. Predicted future temperature and salinity did not affect germination of *A. ostenfeldii*, but affected growth rates, demonstrating their selective effect on the pelagic part of the population when detached from the seed bank. In addition, the importance of resting stages for colonizing new habitats, was stressed by the close relationship found between a recently discovered bloom population in Japan and geographically distant populations of similar habitats. Low genetic diversity indicated a recent introduction, potentially due to anthropogenic dispersal of resting stages.

In conclusion, the seed bank plays a pivotal role for evolution and ecology of *A. ostenfeldii*. It ensures survival of a genetically diverse population, and slows down evolution, by linking contemporary populations to past populations via frequent re-seeding of resting stages. Although selection is buffered by phenotypic plasticity, future temperature and salinity may affect the pelagic part of the population, in the long run. A generalist life cycle of *A. ostenfeldii* and the presence of a seed bank support persistence and potential future temporal and spatial expansion under global change.

Contents

1. INTRODUCTION 7

1.1 Climate change and harmful algal blooms (HABs) 7

1.2 Model organism *Alexandrium ostenfeldii* 8

1.3 Phytoplankton seed banks 12

2. OBJECTIVES 14

3. MATERIAL AND METHODS 15

3.1 Sample collection and culturing 15

3.2 Genotyping 16

3.3 Population genetic analysis 17

3.4 Experiments 18

3.5 Statistical analysis 20

4. MAIN FINDINGS OF THE THESIS 20

4.1 Similar genetic diversity in seed bank and pelagic part of the population 20

4.2 Genetically homogenous pelagic population throughout the season 21

4.3 Frequent sexual reproduction indicated by linkage equilibrium 21

4.4 Overwintering of asexual quiescent cysts without pronounced dormancy 22

4.5 Temperature reduction and combined nutrient limitation trigger cyst formation 23

4.6 Selection happens in the planktonic phase of the life cycle 23

4.7 Plasticity and intraspecific variability aid persistence in fluctuating environment 24

4.8 Japanese isolates form separate clusters 24

5. DISCUSSION 26

5.1 Evolutionary significance of the seed bank 26

5.1.1 Seasonal genotype dynamics and diversity 26

5.1.2 Selection, adaptation and the speed of evolution 27

5.2 Ecological significance of the seed bank 29

5.2.1 Persistence and anchoring 29

5.2.2 Dispersal and expansion under global change 29

6. CONCLUSIONS 31

7. ACKNOWLEDGEMENTS 32

8. REFERENCES 33

1. INTRODUCTION

1.1 Climate change and harmful algal blooms (HABs)

Phytoplankton and climate

Aquatic ecosystems play a central role in bio-geochemical cycling, as they are responsible for nearly half of the planet's carbon sequestration of around 45 gigatons organic carbon per year (Falkowski et al. 1998, Field et al. 1998). Microscopic marine phytoplankton regulates atmospheric CO_2 levels, the export of carbon to the ocean interior and the transfer of carbon to higher trophic levels (Falkowski et al. 1998). In turn, primary production in the oceans depends on geophysical processes, which regulate the mixed-layer depth, nutrient fluxes and food-web structure (Falkowski et al. 1998). Climate change has far reaching consequences for the biosphere and will affect future ecosystems globally (Walther et al. 2002). In the past decades increasing sea surface temperature, stratification and climate variation, which influence the availability of nutrients for phytoplankton growth, were linked to decreasing global net primary production (Behrenfeld et al. 2006). Recent ocean warming has driven changes in productivity, population size, phenology, and community composition of phytoplankton (Thomas et al. 2012 and references therein). Rising temperatures of this century are expected to cause poleward shifts in species' thermal niches and a sharp decline in tropical phytoplankton diversity, in the absence of an evolutionary response (Thomas et al. 2012). Due to their pivotal role in ecosystem functioning and biogeochemistry, phytoplankton has been the focus of global change research in marine ecosystems (Collins et al. 2013).

Global increase of HABs

One consequential effect of ongoing climate change is a greater prevalence of HABs, which have increasingly impacted public health, recreation, tourism, fishery, aquaculture, and ecosystems over the past several decades (Gobler 2020). The societally defined category "harmful algae" comprises toxic phytoplankton species that express toxicity to higher trophic levels, largely fish, shellfish, marine mammals, or humans (Wells et al. 2015). The majority of high-biomass HABs are linked unequivocally to cultural eutrophication, which interacts with other major drivers, such as hydrology, food web interactions, and climate change (Glibert et al. 2018). The anticipated linkages of climate change and HABs are diverse and span from direct effects like the increase in atmospheric and surface water temperature, stratification, altered light conditions, ocean acidification, nutrient shifts and grazing to multiple stressor effects and local human-induced pressures (Hallegraeff 2010). Increased temperature will most certainly alter current spatial and temporal ranges of HAB species: Their geographic domains may expand, contract, or just shift latitudinally and seasonal windows for growth may contract and expand (Hallegraeff 2010). In Northern

European waters pronounced changes in phytoplankton community composition, with an increase of some and decrease of other HAB species, were associated with increased off-shore sea surface temperature and wind speed (Hinder et al. 2012).

HABs in the Baltic Sea

The Baltic Sea is one of the world's largest brackish ecosystems with a strong salinity gradient ranging from >20 practical salinity units (PSU) to almost freshwater concentrations (Meier et al. 2006, BACC author team 2008). Dynamics in the Baltic Sea are controlled by atmospheric forcing due to the low average water depth (54 m) (Leppäranta and Myrberg 2009), which makes it vulnerable to eutrophication, decreasing water quality, loss of biodiversity, decrease in fish stocks, and acidification due to increasing CO_2 levels (Meier et al. 2012a, HELCOM 2018). Effects of changing climate conditions and eutrophication are amongst the most severe anthropogenic stressors deteriorating coastal Baltic ecosystems (Reusch et al. 2018). Due to increased precipitation and freshwater inflow within the catchment area an overall decrease of salinity in the Baltic Sea by 1-3 PSU can be expected in the future (e.g. Meier et al. 2006, 2012b). An increase in sea surface temperature of 2-5 °C is predicted by the end of this century (Graham et al. 2008, Meier et al. 2012a) and warming was found to be the key environmental factor explaining observed long-term changes in plankton communities (Suikkanen et al. 2013). In addition, water temperature in the surface layer was positively correlated with cyanobacteria surface accumulations at the decadal scale (Kahru et al. 2020). In shallow brackish waters of the Baltic Sea and Northern Europe toxin producing dinoflagellates of the genus *Alexandrium* expanded during the past decade and a spreading potential followed by an expansion of blooms under future climate conditions is expected, especially in coastal, shallow waters (e.g. Kremp et al. 2009, 2019).

1.2 Model organism *Alexandrium ostenfeldii*

Characteristics of the genus *Alexandrium*

The dinoflagellate genus *Alexandrium* is globally one of the major HAB genera with respect to the diversity, magnitude and consequences of blooms and has been extensively studied due to public health and ecosystem impacts (Anderson et al. 2012). Species of this genus can produce three different families of toxins and are widely distributed in Northern European waters (Brown et al. 2010, Touzet et al. 2011, Hakanen et al. 2012, Van de Waal et al. 2015, Lewis et al. 2018, Kremp et al. 2019). In addition, a yet incompletely characterized suite of allelochemicals are produced among *Alexandrium* species (Arzul et al. 1999, Tillmann and John 2002, Hakanen et al. 2014, Tillmann et al. 2016). Diverse nutritional strategies include the ability to utilize a range of inorganic and organic nutrient sources and feeding by ingestion of other organisms (Anderson et al. 2012, Blossom et al. 2012). Other characteristics of

this genus are surface-avoiding vertical migration behavior and the ability to produce bioluminescence (Anderson et al. 2012, Le Tortorec et al. 2014, Lindström et al. 2017). Many species of this genus have complex life cycles that include sexuality and often, but not always, cyst formation, which offers considerable ecological advantages (Anderson et al. 2012).

A. ostenfeldii – a globally expanding HAB species

As many other species of this genus, *Alexandrium ostenfeldii* Paulsen (Balech and Tangen 1985), including the heterotypic synonym *A. peruvianum* (Kremp et al., 2014), inhabits cold and temperate waters around the world (Fig. 1, Brandenburg 2019). Typically, the mixotrophic, bioluminescent dinoflagellate occurs at a low cell density together with other dinoflagellates (Jacobson and Anderson 1996, John et al. 2003, Gribble et al. 2005, Le Tortorec et al. 2014). In the Baltic Sea *A. ostenfeldii* started to form dense, recurring blooms with cell densities of up to 1–2 x 10^6 cells L^{-1} in coastal areas in the beginning of this century (Kremp et al. 2009, Hakanen et al. 2012, Le Tortorec et al. 2014), and even denser blooms were recognized in a brackish water creek, connected to the North Sea, with 5.5 x 10^6 cells L^{-1} (Burson et al. 2014). In 2013 a first *A. ostenfeldii* bloom (~ 3 x 10^5 cells L^{-1}) was recognized in Japan in a shallow semi enclosed lagoon (H. Oikawa unpubl. data), but the origin and phylogenetic relationship with other global isolates is so far unknown. A complex phylogenetic structure was found for global isolates earlier, consisting of six distinct, but closely related groups (Van de Waal et al. 2015). The relationships of some groups clearly reflected geographic distribution patterns or habitat preferences, e.g. for cold-water environments (Tillmann et al. 2014). Other geographically distant populations, inhabiting similar ecosystems, seemed to be closely related, which was attributed to recent anthropogenic dispersal (Kremp et al. 2014). Thus, tracking dispersal, mechanisms which facilitate dispersal and phylogenetic relationships of new *A. ostenfeldii* isolates can help to predict and mitigate or even prevent future blooms.

A. ostenfeldii strains are capable of producing allelopathic compounds (Tillmann and John 2002, Tillmann et al. 2007) and different types of neurotoxins, like spirolides (SPX), gymnodimines (GYM) and paralytic shellfish toxins (PST) (Hansen et al. 1992, Cembella et al. 2000, Otero et al. 2010, Tomas et al. 2012, Van de Waal et al. 2015, Zurhelle et al. 2018). Neurotoxins act by reversibly blocking voltage-gated sodium channels in mammals (Catterall 1980), thus inhibiting the transmission of neuronal signals. Toxicity to mammals and negative effects on co-occurring biota have been confirmed for *A. ostenfeldii* (Hansen et al. 1992, Burson et al. 2014).

The life cycle of the species is haplontic, meaning that the motile vegetative cells are haploid (Fig. 2). Sexual reproduction has been documented (Jensen and Moestrup 1997) and resting cyst formation is common. Cyst formation is not an obligate part of sexual reproduction, as in other *Alexandrium* species (Anderson 1998), meaning that

haploid cells and diploid zygotes can form cysts (Figueroa et al., 2008). Short- and long-term resting cysts are difficult to differentiate microscopically, and it is possible that temporary cysts develop into long-term cysts (Fig. 2, 2a-2b, Figueroa et al., 2008). Furthermore *A. ostenfeldii* seems to be mainly heterothallic (i.e. two different mating types are required), but a low level of zygote formation occurred also in clonal strains (Figueroa et al., 2008). A description of the *A. ostenfeldii* life cycle is presently only available from populations of Mediterranean lagoons, one of the many different habitats in which the species lives. Different geographic populations have evolved different life cycle strategies as adaptation to conditions of their respective habitats, as shown for some species (Anderson 1998, Hallegraeff et al. 1998). Triggers for life cycle transitions are known for many well studied *Alexandrium* species (Destombe and Cembella 1990, Anderson 1998, Montresor et al. 2003, Ní Rathaille and Raine 2011, Anglès et al. 2012), but there is a lack of information on Baltic *A. ostenfeldii*. The life cycle of phytoplankton and regulation of transitions from one life stage to another (e.g. germination or cyst formation) play a pivotal role for their ecology, associated food webs and bio-geochemical cycling (Montresor et al. 2003, Persson et al. 2006, Spilling and Lindström 2008). Without knowledge of a species' life cycle, predictions about future bloom formation and modelling are challenging and interpretation of cyst records from the past may be impossible (Ellegaard et al. 2017). Most importantly, different modes of reproduction and cyst formation will affect the rate of sexual reproduction and genetic recombination, which is the basis for genetic diversity and evolution (Rengefors et al. 2017). Thus, gathering basic life history, demographic and ecological data in a context that is useful for evolutionary inference is essential (Collins et al. 2013).

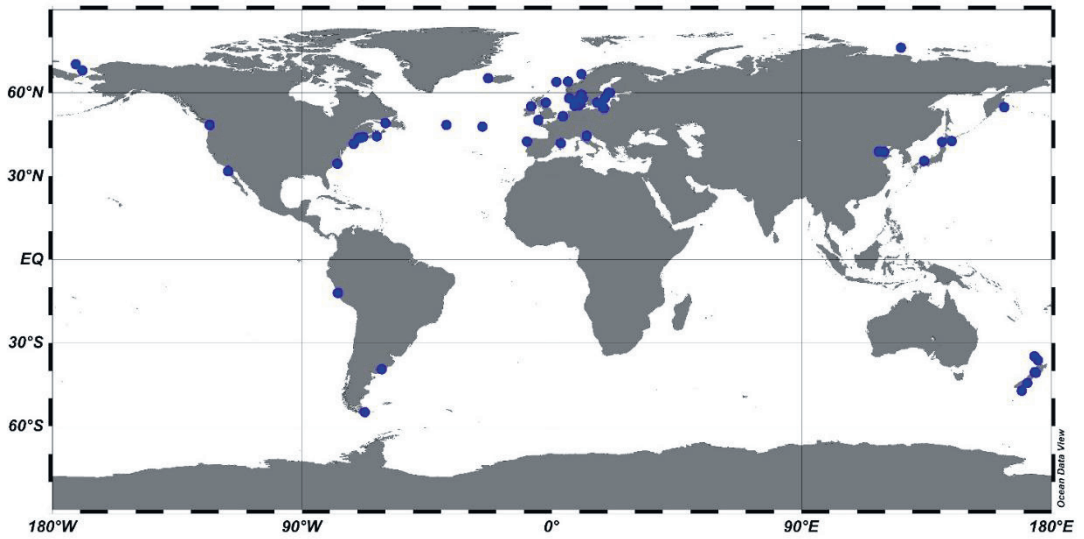


***Fig. 1**. Global distribution of A. ostenfeldii. Map reproduced and updated, with kind permission of Brandenburg (2019).*

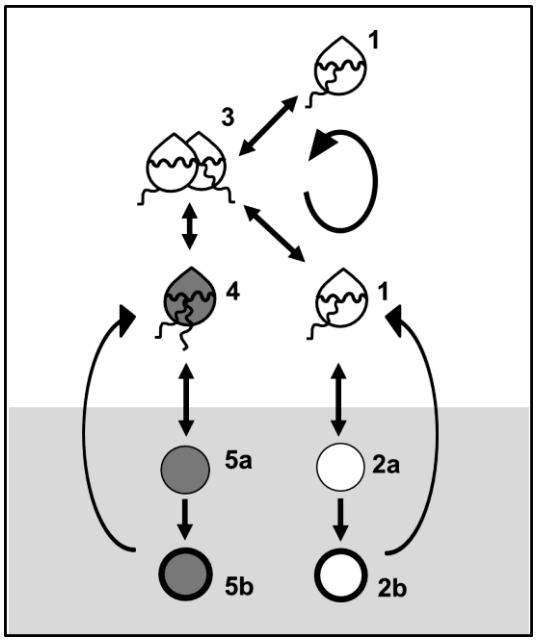


Fig. 2. *Life cycle of Alexandrium, including haploid (empty) and diploid (filled) planktonic cells and resting stages in the sediment (grey area). The motile vegetative cells are haploid (1). Under certain conditions (e.g. stress), vegetative cells can transform into a non-motile temporary cyst (pellicle cyst, 2a), which can quickly switch back to the motile stage when conditions improve (1) or might develop into a long-term resting cyst (2b). In the sexual phase flagellate gametes conjugate (3) and form a diploid planozygote (4), which can transform into a resting cyst (hypnozygote, 5a and b) or undergo meiosis and produce vegetative cells again (adapted from Anderson et al., 2012, Fig. 3).*

Resting stages and mechanisms regulating encystment and germination

Resting stages (cysts or propagules) are non-motile cells with strongly reduced metabolic activity, which typically contain increased amounts of energy-rich storage compounds (Ellegaard and Ribeiro 2018) and can be surrounded by a thick cyst wall (Fig. 2, 2b, 5b), allowing long-term survival in the sediment (Figueroa et al. 2008, Lundholm et al. 2011, Ellegaard and Ribeiro 2018). Resting stages are commonly formed as response to environmental stress, like nutrient depletion, changing temperature, as protection during anoxia and darkness or as a defense mechanism to avoid predation, or infection with viruses and parasites (Toth et al. 2004, Bravo and Figueroa 2014). In addition, resting stage formation can be a passive dispersal strategy for long-distance transport to invade new habitats (Hallegraeff and Bolch 1992). Resting stages can remain encysted, until endogenous and exogenous conditions allow germination. Unfavorable environmental (i.e. exogenous) conditions like temperature, light or oxygen levels can inhibit germination of otherwise competent, "quiescent" cysts (Anderson 1998). In addition, more complex internal regulation mechanisms, like "dormancy" or "secondary dormancy" can suspend germination, despite favorable external conditions (Anderson 1998, Fischer et al. 2018). Such complex mechanisms can prevent germination if suitable conditions don't last long enough to sustain growth and reproduction and might be an adaptation to seasonality at higher latitudes, as demonstrated for other seasonal *Alexandrium* spp., which are dormant for several months (Anderson 1980, Kim et al. 2002, Mardones et al. 2016, Fischer et al. 2018). The dormancy interval can regulate bloom dynamics (Anderson 1998) and has not been studied in Baltic *A. ostenfeldii*.

1.3 Phytoplankton seed banks

Like the seeds of higher plants, resting stages form a seed bank when they accumulate in aquatic sediments and can build up an archive of genetic information when undisturbed layers are buried in the sediment (e.g. Anderson et al., 2012; Ellegaard et al., 2018; Rengefors et al., 2017). The presence of a seed bank, together with reproduction strategies, have several far-reaching consequences for the ecology and evolution of phytoplankton. A seed bank can prolong the persistence of populations and genotypes and can have important implications for community and evolutionary dynamics, like coexistence of species, maintenance of diversity and stability of ecosystems against perturbations (Lennon and Jones, 2011). Especially seed banks of sexually produced resting stages may increase the resistance to change, because such libraries are assumed to be particularly diverse (von Dassow and Montresor 2011, Rengefors et al. 2017). Seed banks can contribute to stability by conserving genetic population structure in the long run (Härnström et al., 2011; Ribeiro et al., 2011) and might facilitate population differentiation in the presence of gene flow (Sundqvist et al., 2018). Active populations in the water column could be "anchored" to historic populations through re-seeding of resting stages (Sundqvist et al. 2018). Thus, resting stages may have long-term evolutionary significance in addition to ensuring seasonal survival (Ellegaard and Ribeiro 2018).

Most importantly, seed banks can affect the rate of evolution: Dormant propagule banks were suggested to either slow down or enhance adaptive evolution, depending on whether the fraction of emerging genotypes is a random or non-random sample of the total gene pool (Hairston Jr and De Stasio Jr 1988). If a random, well mixed fraction of resting stages germinates, evolution can be slowed down due to introduction of maladapted individuals from the past, which have not been exposed to contemporary selection pressures (Hairston Jr and De Stasio Jr 1988). In addition, response to selection is simply slowed down by increased generation overlap, which increases generation time (Yamamichi et al. 2019). Alternatively, the seed bank can speed up evolution, by "migration from the past" if past genotypes, which are well-adapted to current conditions have been preserved and are now re-introduced (Yamamichi et al. 2019). Thus, a genetically diverse seed bank and mechanisms regulating germination may affect a species' ability to adapt to changing environmental conditions.

Although resting stages and seed banks fulfil numerous important roles in ecology and evolution of phytoplankton, a thorough understanding of many processes is still lacking. Cyst formation and germination requirements are unknown for many phytoplankton species, especially because alternative adaptations and life cycle strategies of one and the same species exist in different ecosystems (Hallegraeff et al. 1998). Despite their importance for understanding population genetics and the evolutionary process in phytoplankton, sexual reproduction and dormancy have remained understudied aspects of life history (Rengefors et al. 2017). Thus,

investigating the role of seed banks for toxic phytoplankton species is crucial to advance our knowledge about recurrent blooms and associated threats for affected ecosystems. Studying these aspects becomes even more important in the light of ongoing global change (Chust et al. 2017).

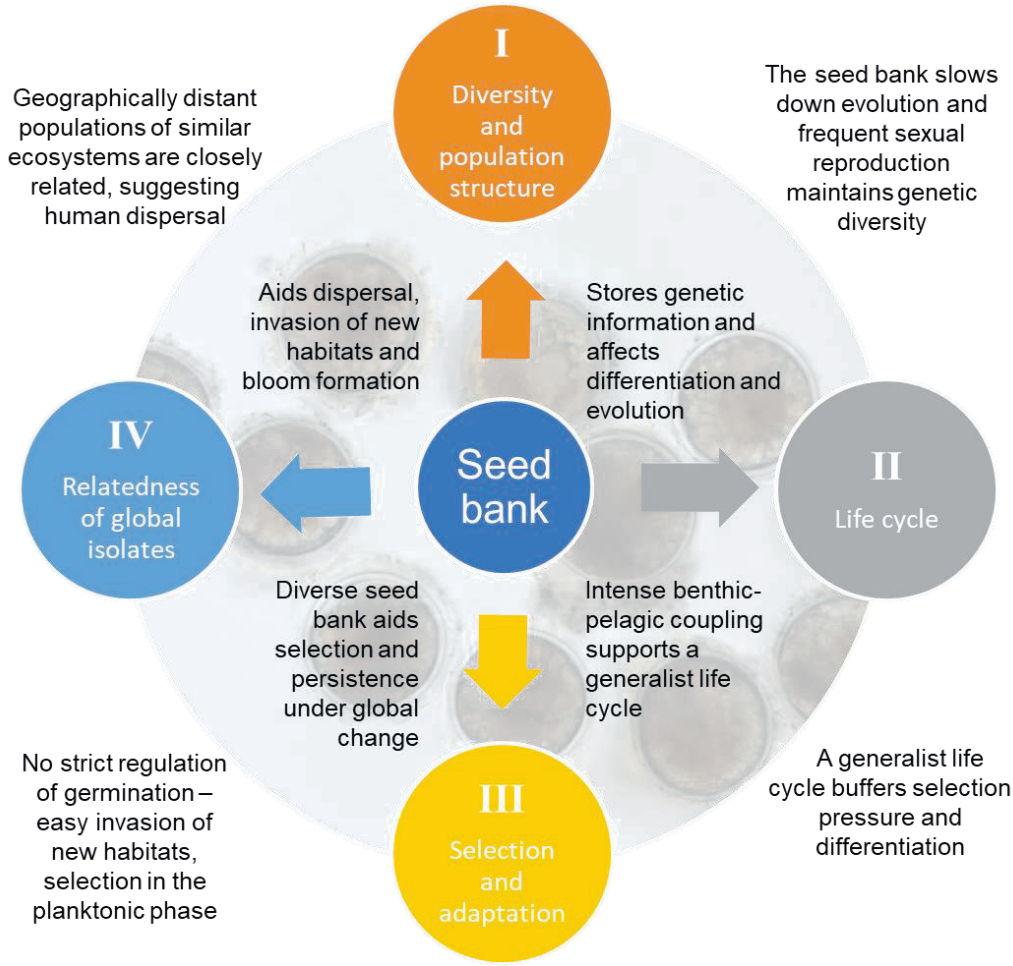


Fig. 3. *Concept of the thesis, including functions of the seed bank relevant for each study (within the large circle) and themed summaries, connecting the four studies to a unified entity (outside the large circle, between the respective studies).*

Investigating genetic diversity and population structure can give insights about the pace of evolution (Fig. 3). Correct interpretation of the population genetic data requires information about reproduction and how the life cycle affects the seed bank diversity. A diverse seed bank may allow selection of the most suitable genotypes from the gene

pool and aid persistence of toxic phytoplankton species, or even expansion. Invasion of new habitats is facilitated by resting stage dispersal and could be fueled by weak germination control. Studying phylogeny of global isolates allows conclusions about dispersal and expansion of *A. ostenfeldii* and may aid the development of measures to prevent further spreading.

2. OBJECTIVES

The overall aim of this study was to investigate the relevance of resting stages and the seed bank for the ecology and evolution of *A. ostenfeldii* and to understand the implications of these findings for the persistence or possible expansion of this HAB species under ongoing climate change.

In this thesis I assessed the genotype diversity of a seed bank and compared it to the pelagic part of the population to determine which part is more diverse and how important the exchange between both habitats is (**Paper I**). I studied the seasonal population structure and frequency of sexual reproduction to gain insights about differentiation and evolutionary dynamics (**Paper I**). I investigated the ecology and life cycle of Baltic Sea *A. ostenfeldii* isolates to find out if this species developed adaptations to local conditions (**Paper II**). To predict how *A. ostenfeldii* will be affected by climate change, I focused on the effect of potential future Baltic Sea salinity and temperature on germination and growth (**Paper III**). Finally, I studied the phylogeny of global isolates to understand the relevance of seed banks for dispersal and bloom formation in new habitats (**Paper IV**).

More specific aims of Papers I-IV were:

- **I.** Assessing the genetic diversity of the seed bank, compared to the bloom population and determine seasonal genotype dynamics.
- **II.** Defining the dormancy interval, delineating triggers for life cycle transitions and assessing how relevant genetic recombination is for the formation of resistant resting cysts.
- **III.** Evaluating if certain clonal lineages are selected from the seed bank by future temperature and salinity conditions and if selection acts at the level of cyst germination or later in the growth phase.
- **IV.** Determining the genetic relation of strains from new bloom locations in Japan with Baltic Sea isolates and other global isolates.

3. MATERIAL AND METHODS

3.1 Sample collection and culturing

Samples for all **Papers** (**I – IV**) were collected in the northern Baltic Sea (Archipelago Sea) at the Föglö archipelago, which is part of the Åland Islands (Fig. 4). This well-known bloom site of *A. ostenfeldii* has been investigated extensively (Kremp et al. 2009, Hakanen et al. 2012). The shallow bay (water depth < 3 m) has a soft muddy bottom, is partly densely vegetated and summer salinity ranges typically between 5 and 6 PSU (practical salinity units). From December to April the bay is usually ice-covered and in summer water temperature can rise to +24 °C. For **Paper IV** two sampling sites in northern and western Japan were studied (Fig. 10 B): Funka Bay, Hokkaido (northern Japan) and Lake Koyama-ike, Tottori Prefecture (western Japan). Lake Koyama-ike has a surface area of 7 km^2 and an average depth of 2.8 m. A salinity of 11.44 PSU and a temperature of 26.23 °C were measured during sampling. Funka Bay is on average 59 m deep (Takahashi et al. 2005) and the average surface salinity is around 32 PSU from March to May (Azumaya et al. 2001)**,** when *A. ostenfeldii* usually occurs.

In 2015 a field survey was carried out at Föglö to study the seasonal genotype dynamics and genetic diversity of *A. ostenfeldii* (**Paper I**). Together with cyst and motile cell samples, several biotic (e.g. phyto- and zooplankton biomass) and abiotic (e.g. temperature, salinity) variables were measured repeatedly throughout the season to capture the environmental heterogeneity and determine potential selection pressures (Table 1). To investigate life cycle aspects of *A. ostenfeldii* (**Paper II**) cysts were sampled in several years and *A. ostenfeldii* cell abundance was recorded together with temperature from May 2010 to April 2011. In addition, continuous temperature measurements at the sediment surface were conducted between April 2017 and May 2017 to define the germination conditions in the field. For **Paper III** cysts were collected only once in September 2015 to conduct germination and transplant experiments. For **Paper IV** motile cells for genotyping were sampled from Lake Koyama-ike, Funka Bay, and Föglö between 2008 and 2013, cysts were sampled in 2015 and environmental variables were recorded during all sampling events. Clonal *A. ostenfeldii* cultures were established from cysts and motile cells isolated from sediment and water samples collected repeatedly between 2008 and 2017 (Table 1). Cyst derived cultures were used in all **Papers** (**I – IV**), for genotyping in **Paper I** and **IV** and for experiments in **Paper II** and **III**. In addition, cultures established from motile cells were genotyped for **Papers I** and **IV**. Sampling procedures, sample processing, culture establishment and culturing conditions are described in detail in the respective papers.

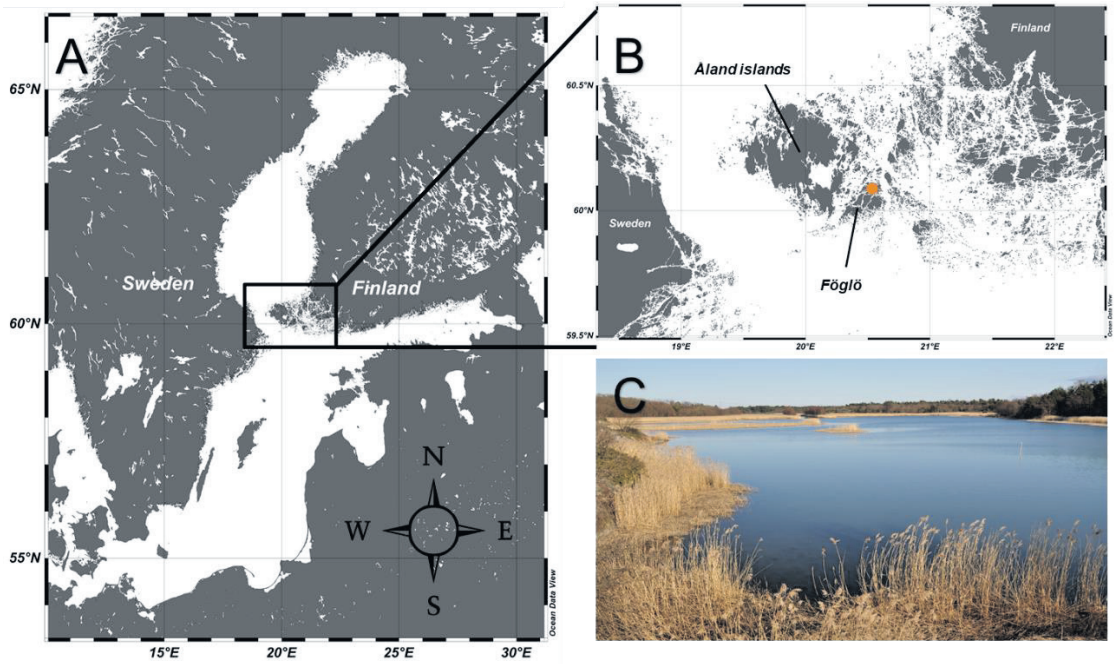


***Fig. 4**. A) Map of the Baltic Sea. B) detailed view of the Föglö archipelago, which is part of the Åland Islands. The sampling location for Papers I – IV is indicated with an orange dot. C) Photograph of the sampling location in May 2017.*

3.2 Genotyping

To study seasonal genotype dynamics and compare the diversity of the benthic and the pelagic Föglö population of *A. ostenfeldii* in **Paper I**, 80 cultures were genetically characterized by restriction site associated DNA sequencing (RAD). Exponentially growing cultures were harvested and concentrated by centrifugation. A detailed description of culturing and harvesting procedures can be found in **Paper I**. A freeze-thawing cycle was applied to support cell break down and DNA was extracted using a cetyltrimethylammonium (CTAP)-based protocol (Dempster et al. 1999), modified as described by Sassenhagen et al. (2015). For RAD library preparations genomic DNA was digested with high-fidelity SbfI (New England Biolabs), applying a protocol modified from Amores et al. (2011) and Etter et al. (2011). Samples were sequenced on a HiSeq2500 system (Illumina), using paired-end 125 bp read length and v4 sequencing chemistry. To identify single nucleotide polymorphisms (SNPs) the RAD data quality was checked with FastQC version 0.11.6 (Babraham Bioinformatics), followed by de-multiplexing, and processing with Stacks software (Catchen et al. 2011, 2013) version 2.41. The Stacks pipeline was run manually after parameter testing to gain maximal coverage. To gain a higher temporal resolution 261 cultures were genotyped additionally with 9 microsatellite markers (MS), developed by (Nagai et al. 2014). Exponentially growing cultures were harvested and frozen at -20 °C and DNA

was extracted in 5% Chelex solution as described by Nagai et al. (2012). Primer pairs and the PCR conditions were described by Nagai et al. (2014) and all primer pairs, except for locus Aosten144 were used for **Paper I**. Gel electrophoresis of PCR products was done with an ABI 3730xl DNA Analyzer (Applied Biosystems) and allele sizes were determined using a 600 LIZ size standard (Applied Biosystems) and GeneMapper v 4.0 (ABI).

The genetic relationship of new isolates from western Japan, with isolates from Föglö and other global isolates was studied in **Paper IV** using MS and ribosomal DNA (rDNA) sequencing. MS were applied to DNA extracted from clonal cultures and single cells, as described above. For phylogenetic analysis the small subunit (SSU), internal transcribed spacer (ITS), and large subunit (LSU) of the rDNA were sequenced after PCR amplification, using previously published forward and reverse primers (Adachi et al. 1994, Takano and Horiguchi 2004, Ki and Han 2007) and primers targeting the end of LSU (Nagai et al. 2010). In addition, a sequence comparison of microsatellite regions was done based on two loci, by PCR amplification and cloning of the amplified fragments into the pGEM-T Easy Vector Systems (Promega) and transformed into *Escherichia coli* following the manufacturer's protocol (Promega 2010).

3.3 Population genetic analysis

For **Paper I** nucleotide diversity (π) was calculated directly in Stacks based on all RAD derived SNPs. Data on the number of RAD sites, variant alleles, and polymorphic sites were also obtained. In the Stacks populations program two filtering options were chosen. To compare the diversity of the benthic and pooled pelagic part of the population, SNP loci shared by two populations and at least 80 % of the individuals were considered (2p). Furthermore, seasonal differentiation was studied with four populations, sharing SNP loci found in at least 80 % of the individuals (4p). Data exploration and all other statistical analysis for RAD and MS were carried out with R version 3.6.1 (R Core Team 2018) and R studio version 1.2.5019 (RStudio Team 2019) using various packages, which are listed in **Paper I**. Basic population metrics of MS data were extracted in R and for both datasets population differentiation (F_{st}) was calculated. In addition, pairwise genetic distances *D* (Jost 2008) and G_{st} (Hedrick 2005) and allelic richness were calculated as measures of genetic differentiation and diversity. Analysis of molecular variance (AMOVA) was performed, followed by a Monte-Carlo test, to check if parts of the population were significantly different from each other. To detect clonal reproduction and investigate if loci are linked, the index of association (I_A) described by Brown et al. (1980) and index $\bar{r}d$ (Agapow and Burt 2001), were calculated for both data sets. Population structure was further investigated using principal components analysis (PCA), a k-means clustering approach and a discriminant analysis of principal components (DAPC) were carried out, followed by DAPC-cross validation.

For **Paper IV** the program MS tools (Park 2001) was used to calculate the number of alleles, allelic frequency, and gene diversity. SSU, ITS and LSU rDNA sequences of different numbers of newly genotyped *A. ostenfeldii* strains were aligned with *Alexandrium* strains downloaded from Genbank. Phylogenetic trees were constructed by using maximum likelihood analysis in MEGA version X (Kumar et al. 2018). Model selection was based on Bayesian Information Criterion (BIC) scores and tree topologies were supported by bootstrap values calculated with 100 replicates.

3.4 Experiments

Laboratory experiments were carried out for **Papers II** and **III** at the Marine Research Centre of the Finnish Environment Institute. In **Paper II** dormancy requirements and seasonal germination were studied by incubating resting cysts, isolated from sediment samples throughout the season, at suitable germination conditions. To define temperatures allowing germination of Baltic *A. ostenfeldii* a temperature gradient experiment was conducted with temperatures ranging from 4 to 20 °C. Single cysts were isolated individually to wells of tissue culture plates and replicates incubated for three weeks at different temperatures. To investigate the relevance of sexual reproduction for cyst formation and determine encystment triggers, experiments were carried out with single clonal cultures and mixes of cultures in tissue culture flasks. Culture medium f/2-Si (Guillard 1975) with reduced nutrient levels (nitrogen, phosphorus or both nutrients reduced to 10 % of the original medium) and reduced temperature (10 °C) served as encystment triggers. Morphologies of lab-produced cysts were compared microscopically, and cyst sizes were measured. Furthermore, the preservation capacity of lab-produced cysts was assessed after one year of storage in the dark at 4 °C, followed by germination experiments.

To study the effect of predicted future temperature and salinity on germination, and detect the life stage susceptible to selection, germination experiments were carried out for **Paper III**. Resting stages were isolated to tissue culture plates containing f/2-Si culture medium with two different salinities (3 and 6 PSU) and incubated at two different temperatures (16 and 20 °C). Germinated motile cells of these experiments were re-isolated to guarantee clonality and used in a follow-up experiment to test the adaptation of *A. ostenfeldii* to germination conditions. Adaptation was tested in reciprocal transplantation experiments, where Chl *a* fluorescence development of the transplanted cultures served as indicator for the growth rate, which was calculated as in Wood et al. (2005). Growth rates were used as as proxy for relative fitness among clones, although fitness might be more complex in slow growing dinoflagellates.

Table 1. *Overview of materials and methods used in* ***Papers I – IV***. *The culture origin indicates if clonal cultures were established from cysts (isolated from sediment) or motile cells (isolated from water samples) to establish clonal cultures and during which month the samples were taken.*

			Culture origin		**Variables recorded**		
Paper	**Location**	**Year**	**Cysts**	**Motile cells**	**Biotic**	**Abiotic**	**Genotyping / experiments**
I	Föglö	2015	Mar, Sep	Jun – Aug	• *A. ostenfeldii* cell abundance • Phytoplankton • Zooplankton • Chl-a	• Toxins • Nutrients • Temperature • Salinity	Genotyping • microsatellites (MS) • restriction site associated DNA sequencing (RAD)
II	Föglö	2010 2011 2015 2016 2017	May – Apr Sep – Apr May	/	• *A. ostenfeldii* cell abundance	• Temperature	Experiments • Dormancy and seasonal germination • Encystment and cyst yield • Temperature requirements for germination • Preservation capacity • Microscopy to compare cyst morphology
III	Föglö	2015	Sep	/	/	/	Experiments • Germination success • Selection at germination level • Adaptation to germination conditions
IV	Lake Koyama-ike, Funka Bay, Föglö	2008 2009 2013 2015	Mar	Mar Mar Oct Aug	• Chl-a	• Salinity • Temperature	Genotyping • MS • rDNA sequencing

3.5 Statistical analysis

Data exploration, basic calculations and plotting of graphs for **Paper II** was done in R (R Core Team 2018), RStudio (RStudio Team 2015) and Sigma Plot V14.0 (Systat Software, San Jose, CA). Smoothed conditional means were added to the sediment surface temperature data in Rstudio using a generalized additive model from the *nlme* package (Pinheiro et al. 2018) as a smoothing method.

For **Paper III** all statistical analyses were performed with R version 3.4.4 (R Core Team 2018) and RStudio (RStudio Team 2015). A Pearson's Chi-squared test was performed to check if temperature or salinity influenced the ratio of germinated cysts. Linear regressions were used to check if the estimated abundance of vegetative cells after germination was related to germination conditions, and if growth rates of the transplant experiment were related to germination or the transplantation conditions. Linear regressions were followed by analysis of variance (ANOVA) and model assumptions were verified by plotting residuals *versus* fitted values.

The origin of northern Japanese *A. ostenfeldii* isolates in **Paper IV** was modeled using a high resolution (1/50°) ocean model (Kuroda et al. 2014). A backward particle-tracking model configured for Regional Ocean Modelling System (Shchepetkin and McWilliams 2003) was applied, in a depth range of 5 and 30 m, where *A. ostenfeldii* cells were detected during 2008 and 2009. The simulation periods were 60 and 30 days, covering the period from mid-January/February to mid-March.

4. MAIN FINDINGS OF THE THESIS

4.1 Similar genetic diversity in seed bank and pelagic part of the population

Both genetic markers revealed high clonal diversity (Fig. 5), combined with low to intermediate gene diversity (depending on the marker) and allelic richness of the locally restricted *A. ostenfeldii* population in the Baltic Sea (**Paper I**). In total 261 strains were successfully characterized with MS, based on 9 loci and 54 alleles. With RAD 78 strains were successfully genotyped and for comparing the benthic and pelagic population 415 polymorphic RAD loci were utilized after SNP filtering (2p). Both genetic markers indicated that the genetic diversity (H_e) and mean allelic richness of the benthic and pelagic population are very similar (**Paper I,** Table 1 and 2). Furthermore, AMOVA results showed no population differentiation between the benthic and pelagic population (**Paper I**, Table S3).

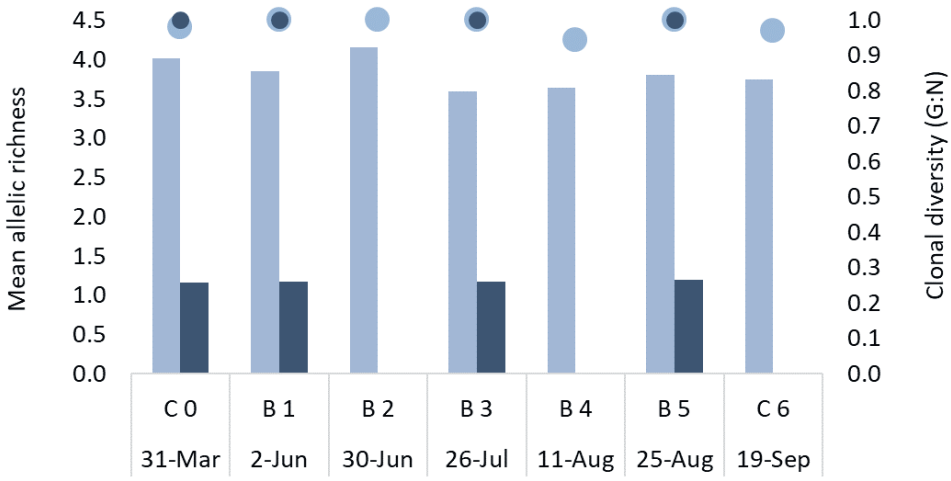


Fig. 5 *Mean allelic richness of benthic (C0, C6) and pelagic (B1-B5) parts of the Föglö population, based on MS (light blue bars) and RAD (dark blue bars) and clonal diversity (ratio of the number of unique genotypes (G) to the total number of isolates genotyped (N)) based on MS (light blue dots) and RAD (dark blue dots) during 2015.*

4.2 Genetically homogenous pelagic population throughout the season

A field survey, carried out in 2015 at the Föglö archipelago, revealed a succession of potential selection pressures (**Paper I**), like low temperature, high abundance of zooplankton, low nutrient levels and high phytoplankton biomass. Pairwise genetic distances F_{st} shown in Fig. 6 (Nei, 1973), D (Jost 2008) and G_{st} (Hedrick 2005) based on MS data indicated weak, non-significant differentiation between population pairs (**Paper I**). The RAD dataset included 246 polymorphic loci after SNP filtering (4p) and RAD-based pairwise genetic distances indicated very little (F_{st}, Nei (1973) and D (Jost 2008)) or no differentiation (G_{st}, Hedrick, 2005) between all population pairs. The pairwise genetic distance results were supported by AMOVA results, pointing at a panmictic population during the entire season (**Paper I**, Table S2).

4.3 Frequent sexual reproduction indicated by linkage equilibrium

To determine the importance of sexual reproduction throughout the season for benthic and pelagic parts of the population, the index of association (I_A) and the index of association adjusted for the number of loci ($\bar{r}d$) were calculated in **Paper I**. For clonal populations significant disequilibrium is expected due to linkage among loci, whereas for sexually reproducing populations no linkage among loci is expected. Based on MS and RAD data, non-significant I_A and $\bar{r}d$ values close to zero indicated linkage equilibrium for all parts of the population, sampled at different dates, except of bloom population B3 (RAD), where $\bar{r}d$ was very low (-0.01) but significantly different from a random distribution ($p < 0.05$).

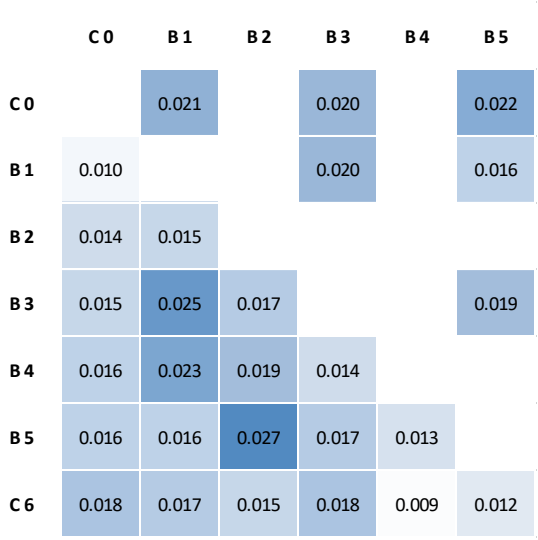
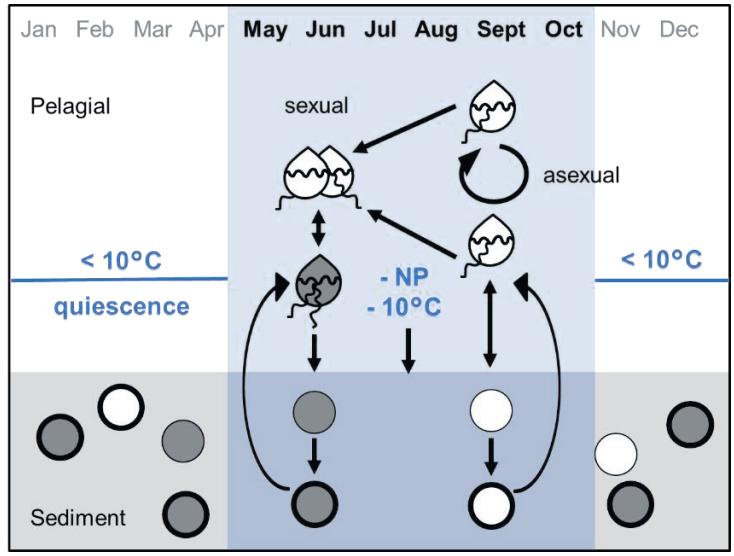


Fig. 6. *Heatmap of pairwise genetic distances for F_{st} values, based on MS below the diagonal and RAD above the diagonal (Nei, 1973). C refers to cyst populations, B refers to bloom populations, numbers indicate different time points. Darker color indicates stronger differentiation and p-values obtained with a Monte-Carlo test after 999 permutations indicated no significant difference for any pairwise comparisons.*

4.4 Overwintering of asexual quiescent cysts without pronounced dormancy

Germination experiments with cysts sampled repeatedly during one season and laboratory produced cysts showed that *A. ostenfeldii* can germinate all year long after newly formed cysts have undergone a short maturation period of around one month, at favorable growth conditions (**Paper II**). As indicated in Fig. **7** no pronounced dormancy period was found, and quiescence was terminated by a temperature increase above 10 °C. In addition, sexual heterothallic reproduction is not required for overwintering, but it increased germination capacity and germling survival after a resting period.

Fig. 7. *Graphical summary of **Paper II**: The growth season (blue shade) for Baltic A. ostenfeldii is restricted by water temperature below 10 °C. Sexual (filled circles) and asexual reproduction (empty circles) allow over-wintering. Sexual reproduction increases germination capacity after a resting period (more filled circles). Combined nutrient reduction (-NP) and temperature reduction (-10 °C) trigger cyst formation most efficiently and different encystment triggers resulted in various cyst morphologies (e.g. thin- and thick-walled cysts represented by thin and thick lines around circles).*



4.5 Temperature reduction and combined nutrient limitation trigger cyst formation

Encystment experiments with cultured *A. ostenfeldii* isolates showed that cyst formation is regulated by multiple factors, resulting in substantial variation of cyst yield, cyst morphology, cyst preservation and germination capacity (**Paper II**). Combined nitrogen and phosphorus limitation (-NP) and temperature reduction (-10 °C) resulted in highest cyst yields in clonal cultures and mixes of two and five strains. Moreover, cyst formation induced by combined nitrogen and phosphorus limitation supported the preservation capacity of sexually produced cysts (Fig. **7**).

4.6 Selection happens in the planktonic phase of the life cycle

Germination experiments with 378 single cysts, carried out to define the life stage susceptible to selection, clearly demonstrated that predicted future temperature and salinity have no effect on germination in the narrow range tested (**Paper III**). In contrast, both factors affected vegetative growth after germination: Higher temperature accelerated and lower salinity decelerated growth significantly, compared to control conditions (Fig. 8). In addition, temperature and salinity had opposing effects on growth and balanced each other's effect when combined.

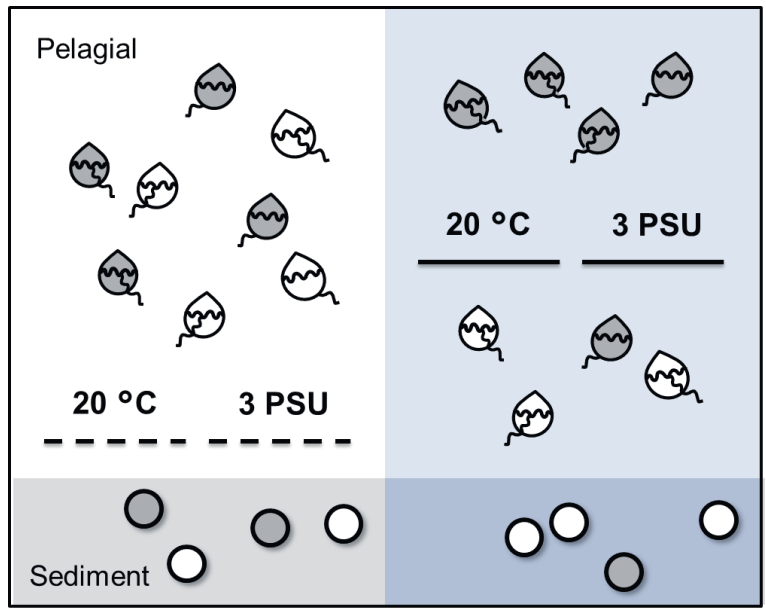


***Fig. 8**. Summarized results of germination experiments in **Paper III**. Predicted future temperature (20 °C) and salinity (3 PSU) had no significant effect on germination (left side) and thus did not represent an environmental filter (horizontal dashed line), but both environmental variables significantly affected the growth rate in the pelagic phase (blue shade, right side) – representing environmental filters (horizontal lines) – compared to control conditions (16°C, 6 PSU). Favored clonal lineages (cysts and motile cells) are filled with grey.*

4.7 Plasticity and intraspecific variability aid persistence in fluctuating environment

Transplantation experiments revealed that *A. ostenfeldii* strains were not adapted to germination conditions, but able to adjust to temperature and salinity different from their germination conditions (**Paper III**). Transplanted strains even outperformed non-transplanted control strains and a high variability of strain specific growth rates occurred. Furthermore, significantly higher growth rates were observed at higher temperature (Fig. 9).

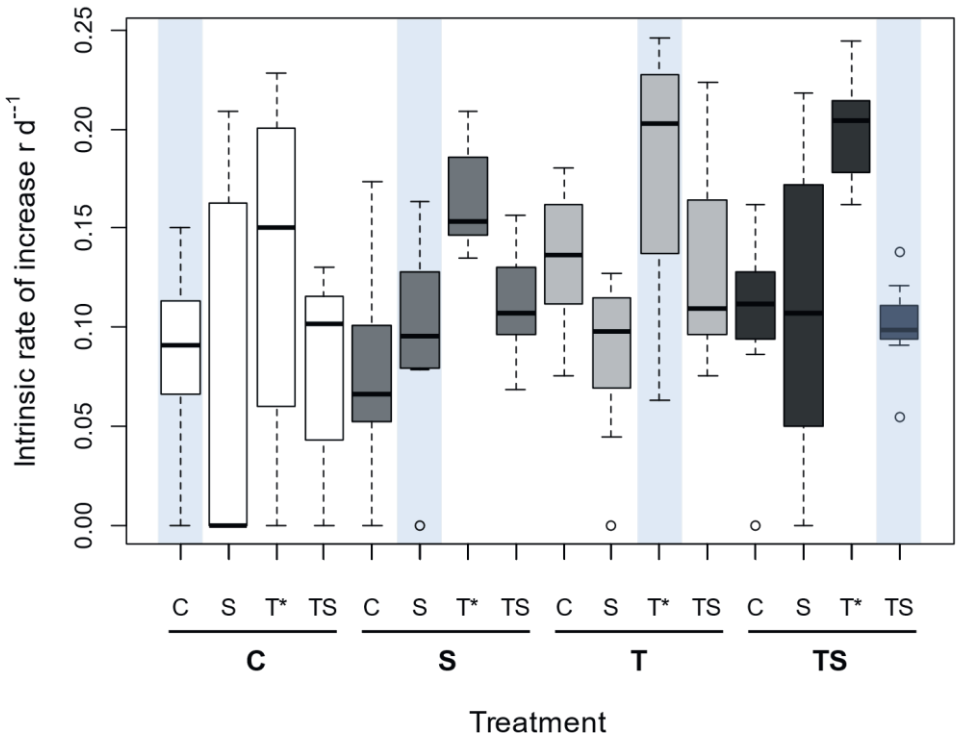


Fig. 9. *Growth rates of strains transplanted from germination conditions (bold letters, color of the bars) reciprocally to growth conditions: Control (C): 16 °C, 6 PSU; high temperature (T): 20 °C, 6 PSU; low salinity (S): 16 °C, 3 PSU; high temperature combined with low salinity (TS): 20 °C, 3 PSU; n = 8 for all treatments (except for T and TS germinated under condition TS, n = 7). Blue shades indicate equal germination and growth conditions. Asterisks indicate treatments significantly different from the control ($p < 0.05$).*

4.8 Japanese isolates form separate clusters

As revealed from phylogenetic analysis of SSU, ITS and LSU rDNA regions, *A. ostenfeldii* isolates from the new bloom site in western Japan did not cluster together with isolates from northern Japan (**Paper IV**). Instead, isolates from western Japan mainly clustered with Baltic Sea isolates and other isolates from shallow and

productive coastal areas with low salinity (Fig. 10, group 1). Genotyping with MS revealed low genetic diversity and was only successful in two out of ten microsatellite loci in western Japanese samples due to lack of PCR amplification.

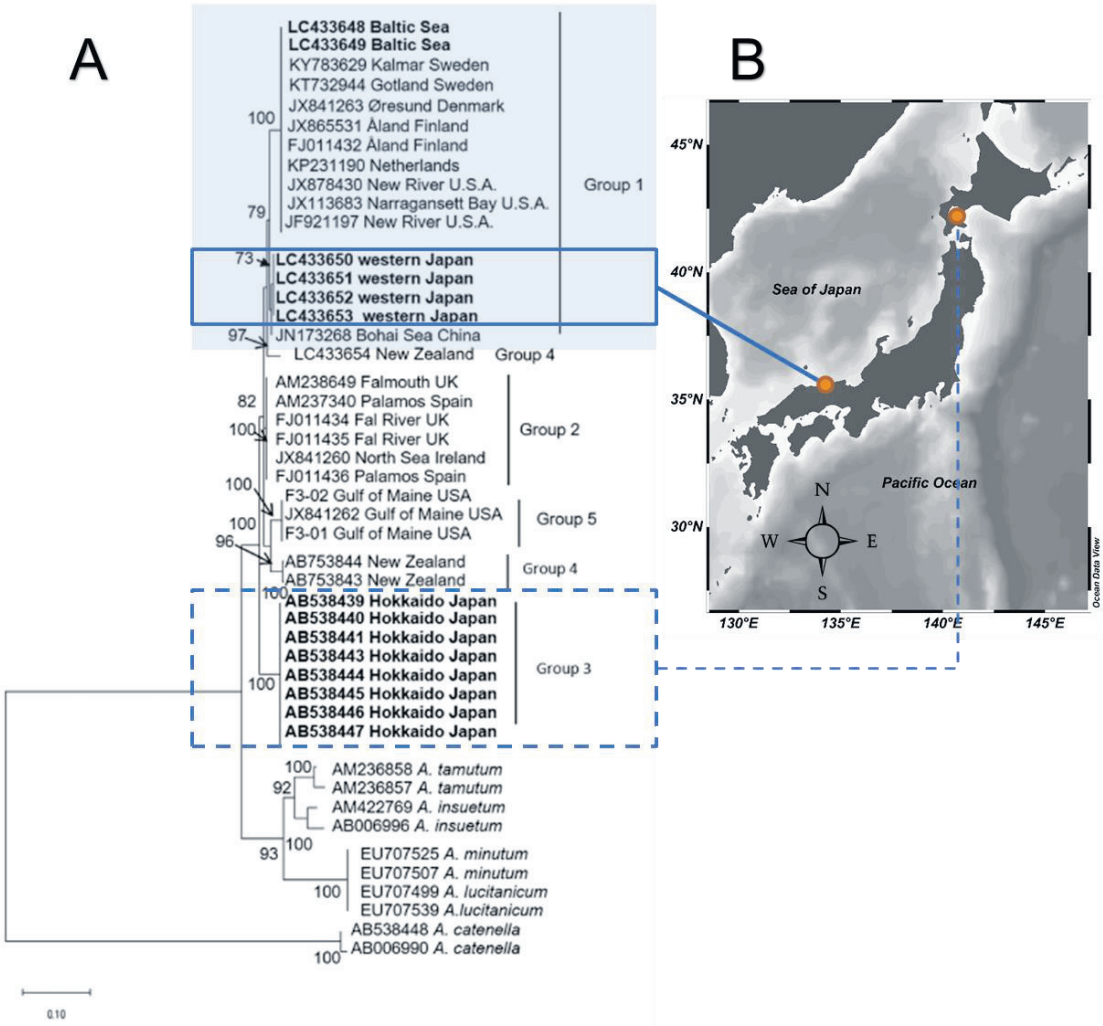


Fig. 10. *A) Maximum-likelihood tree based on the ITS alignment (525 bp, with insertion/deletions) including 46 sequences and A. catenella serving as an outgroup. Bootstrap values >50% are shown. Group assignments based on Kremp et al. (2014). The blue shade highlights group 1 and the blue boxes surround strains from Japan. B) Location of both study sites (orange dots) in western and northern (Hokkaido) Japan.*

5. DISCUSSION

5.1 Evolutionary significance of the seed bank

5.1.1 Seasonal genotype dynamics and diversity

Paper I of this thesis revealed similar diversity levels in the benthic and the pelagic part of the *A. ostenfeldii* population at Föglö, which contrasts with the common expectation of increased diversity of seed banks (Rengefors et al. 2017) and zooplankton egg banks (Brendonck and De Meester 2003). To date only one other phytoplankton study compared genetic diversity of the benthic and pelagic parts of a diatom population and found that diversity was not significantly different (Godhe and Härnström 2010), which supports the results of **Paper I**. The expectation to find an increased seed bank diversity – or reduced diversity of the pelagic part of the population – is based on two assumptions. The first assumption is that propagation in the pelagic phase is based on asexual reproduction (resulting in increased linkage of loci) and cyst formation is associated with sexual heterothallic reproduction. The second assumption is that only a subset of resting stages germinates at the beginning of the season and sexual reproduction along with cyst formation terminate vegetative growth at the end of the season. Results of this thesis suggest different dynamics for *A. ostenfeldii* growing in shallow habitats. Linkage equilibrium indicated by I_A and $\bar{r}d$ values, derived from two types of genetic markers in **Paper I**, suggests that sexual reproduction happens not only at the end of the season, but more frequently and contributes to high genetic diversity in the pelagic phase. Experimental results of **Paper II** confirmed that germination is possible throughout the season, which probably allows regular exchange of the pelagic part of the population with the seed bank. This assumption is supported by the finding that various triggers induced cyst formation, which could intensify benthic-pelagic coupling. Another important result of **Paper II** is the successful germination of resting stages formed in clonal cultures after overwintering. Those resting stages were formed either asexually or after sexual homothallic reproduction. A large contribution of asexual (i.e. haploid) resting stages would lower the diversity of the seed bank, compared to a seed bank consisting exclusively of sexually produced resting stages. Thus, a balance between asexual and sexual reproduction is required to maintain the diversity of the population. In addition, frequent exchange between benthic and pelagic parts of the population harmonizes high clonal diversity in both habitats.

The described dynamics have important consequences for seasonal population structure. Results of **Paper I** revealed very little, non-significant population structure between the temporally divided parts of the pelagic population in 2015, despite a succession of potentially strong selection pressures. Salinity, temperature, nutrient availability and grazing affected the magnitude and duration of *A. ostenfeldii* blooms in the Netherlands (Brandenburg et al., 2017), indicating that those environmental

factors can potentially exert selection pressure. One study on *A. fundyense* from a shallow pond reported temporal genetic intra- and inter-bloom differentiation based on four microsatellite loci and explained it with selection pressure exerted from parasitism and zooplankton grazing (Richlen et al. 2012). The same study found genetic differentiation between shallow- and deep-water fractions of a population, explained by behavior and hydrographic factors, which limited exchange between population parts (Richlen et al. 2012). Genetic intra- and inter-bloom differentiation of an *A. minutum* population potentially resulted from fluctuating temperatures, but the authors suggested that sexual reproduction and/or migration could regularly erase any genetic structure produced within estuaries (Dia et al. 2014). Rapid temporal and spatial genetic differentiation of *A. catenella* populations was likely associated with natural selection through environmental conditions such as nutrient concentrations (Gao et al. 2019). Stronger selection presumably also led to linkage disequilibrium, lower allelic richness and reduced gene diversity in a pelagic population part of the diatom *Skeletonema marinoi* (Godhe and Härnström 2010). The above-mentioned studies investigated deeper sites (e.g. Gao et al. 2019) and larger temporal scales (differentiation between years) and different species, which might partly explain the detection of population structure. Genetic differentiation of populations requires natural selection, mutation or genetic drift, in combination with limited gene flow (Rengefors et al. 2017), which could be realized if there is very little exchange of the pelagic part of the population with the seed bank.

5.1.2 Selection, adaptation and the speed of evolution

In **Paper III** of this thesis selection affected the pelagic part of the population when it was detached from the seed bank in transplantation experiments, where predicted future temperature and salinity had a significant effect on growth rates. These results suggest that favored clonal lineages can grow faster, multiply their genetic material quicker and potentially contribute to a larger extent to the gene pool of the pelagic population, if there is no exchange with the seed bank. Subsequently, differentiation and population structure could emerge. The absence of seasonal population structure, as indicated by F_{st} values and AMOVA results in **Paper I**, probably results from similar processes as the harmonized diversity of the seed bank and the pelagic part of the population: Despite natural selection, the dominance of superior clonal lineages was likely prohibited due to frequent sexual reproduction and exchange of the pelagic part of the population with the seed bank. Further possible explanations are that the dynamic environment never allows dominance of one or a few clones, analogous to the Intermediate Disturbance Hypothesis (Connell 1978) and that population differentiation was prevented by high plasticity, which counteracts selection for different ecotypes / subpopulations. Other relevant explanations might be the control of dinoflagellate blooms by host-specific parasites (Chambouvet et al. 2008) or host-specific viruses and size-selective grazers that are selectively "killing the winner" and thereby maintain equilibrium diversity (Thingstad 2000, Winter 2012). Additionally,

the exchange between the pelagic and the benthic habitat is probably intensified by the ability of *A. ostenfeldii* to form temporary resting stages and the short resting period of newly formed cysts, as documented in **Paper II**.

Dormant resting stage formation is beneficial in unpredictable environments, and the ability of an individual to exist in an active or dormant state is an example of phenotypic plasticity (Lennon and Jones 2011). Phenotypic plasticity seems to be favored in fluctuating environments (Stomp et al. 2008) and can weaken the effect of natural selection on individuals and their associate traits (Chevin et al. 2010). This could explain the plastic growth rate responses measured during transplantation experiments of **Paper III** and the large phenotypic plasticity observed in life cycle transitions of **Paper II**. Alternatively, there can be strong selection on phenotypic plasticity, because maintaining such plasticity is assumed to be costly (Lennon and Jones 2011). Thus, the formation of temporary *A. ostenfeldii* resting stages could be a compromise to ensure persistence in a fluctuating environment, and at the same time reduce the energetic costs associated with the formation of dormant long-term resting stages. If the ability to enter a dormant state is retained depends amongst others on the amount of environmental variation over extended periods of time, its effect on fitness, the mutation rate, and the effective size of the population (Shoemaker and Lennon 2018).

Since population differentiation did not arise within one growth season (**Paper I**), it may be assumed that evolution is slowed down by the seed bank, because resting periods lengthen the generation time of microorganisms (Lennon and Jones 2011). This was confirmed by studies on bacterial evolution, demonstrating that the evolutionary rate of endospore-forming bacteria may increase when a lineage loses the ability to form resting stages, but may decline as the average time spent in the seed bank increased (Shoemaker and Lennon 2018). The evolutionary role of seed banks is complex and depends amongst others on germination dynamics of the contained resting stages, as suggested for zooplankton (Hairston Jr and De Stasio Jr 1988). If the fraction of emerging genotypes is a mixed sample of the total gene pool, including resting stages formed in several years, then selection can be slowed down by the seed bank (Hairston Jr and De Stasio Jr 1988). Alternatively, the seed bank allows rapid selection if mainly cysts from the previous season germinate (Hairston Jr and De Stasio Jr 1988). For *A. ostenfeldii* overlapping generations probably complicate dynamics compared to zooplankton, which makes only two generations per season (Hairston Jr and De Stasio Jr 1988). However, the large clonal diversity of *A. ostenfeldii* confirmed in **Paper I** most likely facilitates evolutionary adaptation if selection pressures last longer, become more intense or benthic-pelagic coupling is reduced. Genotypes with highly variable competitive abilities may evolve in timescales significantly shorter than climate change (Bach et al. 2018), thus, global change related environmental variation will most likely not compromise the occurrence of this species in the Baltic Sea in the future.

5.2 Ecological significance of the seed bank

5.2.1 Persistence and anchoring

The seed bank supports persistence of genotypes in general (Lennon and Jones 2011) and the advantage of resting stages has been suggested to explain the high survival rate of coastal phytoplankton groups relative to the extinction of many oceanic phytoplankton species at mass extinction events in the past (Kitchell et al. 1986, Ribeiro et al. 2011). Seed banks were proposed to "anchor" active populations in the water column to historic populations through re-seeding of resting stages (Sundqvist et al. 2018). Successful revival of up to a century old dinoflagellate resting stages from layered sediment cores demonstrated that re-seeding from very old populations is theoretically possible (Lundholm et al. 2011, Ribeiro et al. 2011). It is likely that resting stages produced by *A. ostenfeldii* serve as long-term survival strategy, but in shallow habitats like Föglö, the formation of short-term resting stages, which allow seasonal survival, is maybe more important. **Paper II** of this thesis indicates that short-term resting stages can be formed under various conditions, which emphasizes their importance for the persistence of the species. **Paper II** provides evidence that resting stages produced without heterothallic sexual reproduction can germinate after several months of cold storage, indicating their relevance for overwintering, which contrasts earlier assumptions for this species (Jensen and Moestrup 1997). At the same time results of **Paper II** highlight the dual functionality of cysts, which is their ability to quickly resume vegetative growth but also to survive for prolonged periods in the dark as non-motile stages (Bravo and Figueroa 2014).

Temperature gradient experiments of **Paper II** revealed that germination of *A. ostenfeldii* is possible within a broad range of temperatures (10 – 24 °C) which currently allows germination and vegetative growth from April to October in the Baltic Sea. Future increases in temperature may increase the length of the season both for vegetative growth and inoculation from the seed bank. It is a common expectation that seasonal windows of opportunity for HABs will expand at higher latitudes due to global change (Wells et al. 2015). In the Gulf of Finland, a modelling study revealed that temperature and life cycle-related processes explain the relative increase of dinoflagellates compared to diatoms, due to warming in the past and the advantage of a large inoculum from the seed bank (Lee et al. 2018). Warming over 30 years has enabled a head start of dinoflagellates by reducing the time lag between germination of resting stages from a seed bank and growth of vegetative cells (Lee et al. 2018).

5.2.2 Dispersal and expansion under global change

Seed banks play an important role for inoculating large HABs (Anderson et al. 2012) and in addition to temporal expansion, HAB geographical domains are expected to expand in some cases due to climate change (Wells et al. 2015). Extension of

geographic ranges and colonization of new habitats can be facilitated by the transport of cysts from one location to another in several ways: Natural discharge of water by currents (Park et al. 2018, Gao et al. 2019), transport by waterfowl (Tesson et al., 2018) or human-assisted dispersal, e.g. by ships' ballast water (Hallegraeff and Bolch, 1991) or drifting plastic debris (Masó et al. 2003), may distribute resting stages. Whether colonization of new habitats is successful or not will depend on local environmental conditions, including interactions with endemic species (Azanza et al. 2018). Colonization of new habitats might be limited, if resident populations outcompete invaders during germination, thus the ability to germinate under different conditions was suggested to explain the expansion of freshwater phytoplankton in northern Europe (Sassenhagen et al. 2015a). Similarly, the broad temperature window for germination of *A. ostenfeldii* reported in **Paper II** of this thesis might aid its successful invasion of new habitats. Once invasion is accomplished, non-native species may occur in low abundances until environmental change favors particular species (Smayda 2007). For example, a broad scale environmental change in Northland, New Zealand was associated with a new major bloom of the toxic dinoflagellate *Gymnodinium catenatum* (Irwin et al. 2003). Resting stages of this species were found in sediment cores, evidencing its occurrence much earlier than the first major bloom (Irwin et al. 2003). Likewise, the first *A. ostenfeldii* bloom record from western Japan in **Paper IV**, could have resulted from a recent broad scale environmental change, since a major salinity increase happened after the opening of flood gates (Nomura et al. 2009). A longer presence of *A. ostenfeldii* in Lake Koyama-ike cannot be excluded, but the low diversity of isolates from western Japan and a lack of previous records of this species in the area indicate a recent introduction. This hypothesis is supported by phylogenetic analysis of global *A. ostenfeldii* isolates of **Paper IV**, suggesting that new isolates from western Japan were more closely related to Baltic Sea isolates and one isolate from Bohai Sea (China). The distant relationship between *A. ostenfeldii* isolates from western and northern Japan could be explained by habitat differences and the geographic isolation of the northern Japanese population, revealed by a high-resolution ocean model (**Paper IV**). At the same time anthropogenic introduction of *A. ostenfeldii* from China to western Japan might be possible via cargo exchange between China, Korea and Japan, which emphasizes the importance of resting stages for dispersal. Seed banks could promote anthropogenic cyst dispersal even further, if natural ecosystems are disturbed or manipulated without prior knowledge of the ecosystem and the life cycle of occurring species.

In the last decades a clear increase of HAB events was recognized around the world, either in the form of geographical expansion of toxic or harmful species, or in the form of an increased diversity of toxic species and toxic compounds (Lassus et al. 2016). The observed trends of range expansion and increased frequency in coastal areas are attributed partly to the effects of ocean warming, marine heatwaves, oxygen loss, eutrophication and pollution (Gobler 2020). However, results of a long-term study challenged the view that HAB species have increased in frequency and intensity and

have become more widespread in recent decades in Northern European waters (Hinder et al. 2012). Interactive effects of increased sea surface temperature and wind speed lead to a decline of some and increase of other HAB species over the last 50 years (Hinder et al. 2012). Thus, despite the well-documented global trends in HABs, being promoted anthropogenically, individual events are driven by local, regional, and global drivers, making it crucial to evaluate the conditions and responses at appropriate scales (Gobler 2020).

6. CONCLUSIONS

As demonstrated in this thesis the seed bank plays a pivotal role for evolution and ecology of *A. ostenfeldii*. Most importantly, it ensures survival of a genetically diverse population at different time scales. Short-term survival during seasonal environmental fluctuations and overwintering is guaranteed by formation of different types of cysts. In the long-term the seed bank buffers the population against rapid directional selection under gene flow, which stabilizes the population against environmental instability. Evolution is slowed down by the seed bank, because contemporary populations are connected to ancient populations via random re-seeding of resting stages and gene flow between the seed bank and the pelagic part of the population is assured by frequent sexual reproduction and benthic-pelagic coupling. These two mechanisms are important for maintaining a large clonal diversity, which will most likely aid persistence of this species under global change. Future temperature and salinity can affect the pelagic part of the population if it is detached from the seed bank, but selection might be neutralized by a large phenotypic plasticity. Persistence and potential future expansion might be supported by the generalist life cycle of *A. ostenfeldii*, including flexible formation of cysts, with or without sexual heterothallic reproduction, induced by various triggers, and no strict germination control. A short dormancy period combined with a wide temperature window for germination allows occurrence of motile cells from May to October in the Baltic Sea. This seasonal growth window will most likely expand in the future. Furthermore, the seed bank may aid dispersal of *A. ostenfeldii* resting stages and invasion of new habitats, like lake Koyama-ike in western Japan. During suboptimal environmental conditions invaders may occur in low abundance and remain unrecognized until conditions (e.g. salinity) change and allow bloom formation, potentially supported by seed banks.

7. ACKNOWLEDGEMENTS

First and foremost, I want to thank my supervisors Anke and Sanna. Without you I would have never gotten the opportunity to come to Finland, become part of the Reservoir project and the international research community. Your doors were always open, and I am truly grateful for all the advice and support I got. Thank you, Karin, your support was indispensable for Paper I of this thesis, from visiting your lab in Lund to many fruitful online discussions about the RAD data analysis and your contribution on writing the Paper. Satoshi, I am very grateful for my research visit to Japan and that you taught me how to do genotyping with microsatellites. Sirje, I highly appreciated your efforts on unravelling *A. ostenfeldii* relationships in Japan and the good communication throughout the years. Special thanks go to Conny, you helped me at different times of my PhD, from getting started in the lab in Kumpula and data analysis workshops in Stockholm and Turku to your valuable comments on Paper I. Thanks to all my co-authors for their important contribution on the respective papers. I would also like to thank my pre-examiners Peter von Dassow and Raphaele Siano for taking the time to review my thesis and the helpful comments I got.

The support of some people, not named as co-authors was indispensable for the success of my work: Johanna, I am extremely thankful for all your help in the lab throughout the years - without you many things would have been impossible. Similarly, Marie Svensson's help was essential in the lab in Lund and I am very grateful for your patience. I also want to thank Dora Čertnerová for helping me to get started with my RAD library preparation and Ritsuko Kubota for her assistance during the microsatellite work in Japan. I want to acknowledge Riina Klais, for her flexibility and advise on the statistics of Paper II. Moreover, a big thank you goes to Johan Franzén, for assisting many times with the sampling and his great hospitality during our visits at Föglö. Kaisu Jussila is thanked for her fantastic support of the lab work for Paper II in Viikki and for her flexibility to support my bioturbation experiments at the Zoological Station in Tvärminne. We had a fantastic, but also extremely challenging time there and learned a lot about how not to carry out bioturbation experiments. Joanna Norkko supported my endeavor in Tvärminne, for which I am very grateful. Joanna was also part of my thesis advisory committee, and together with Outi Setälä and Harri Kankaanpää I want to acknowledge your advice and time spent on our regular advisory committee meetings.

Furthermore, I would like to express my gratitude to the leaders of the Marine Ecosystems and Modelling group (MEM) at the Marine Research Center of SYKE: Timo Tamminen, Risto Lignell, Harri Kuosa and Jonna Piiparinen: thanks for all the administrative work and making me feel part of the Marine Resarch Center (MRC). Special thanks to Risto for your good advice during difficult times. I also want to thank my MEM colleagues for the great atmosphere in our group and to all other colleagues at MRC who were not too shy to have lunch or coffee breaks in English. Thanks to Elina and Eeva for helping me to solve some coding challenges, to Tere for introducing me to the large circle of international friends in Finland. Thanks to Heidi, Riikka,

Heikki, Danielle, Mari, Lumi, Kristian, Seppo, Kaarina, Kirsten and many other colleagues for listening to my complaints when facing challenges at work and for the inspiring discussions during seminars. I am also very grateful for the exciting conference experiences and discussions with Henna Savela, Ingrid Sassenhagen and Karen Brandenburg, who kindly provided data for Figure 1 of this thesis.
Thanks to all my friends for distracting me from work at times and keeping me positive and sporty due to countless hockey and football games or other social activities. Three friends, who supported my wellbeing, and thereby the progress of my PhD enormously, are Louise, Katja and Katrin. Thank you very much for our encouraging scientific and nonscientific discussions. Louise, I enjoyed our, sometimes heated, debates as well and particularly our regular supervision meetings!
My biggest thanks go to my family: Your support is the basis my achievements. I couldn't have reached my aims without the endless care and love of my parents Edi and Edith. Danke, ihr seid die Besten!!! I also want to acknowledge my brother Thomas and my sister in law, Regina, for helping with sampling and your support during challenging times. I am grateful to my aunt Ingrid and uncle Peter, who were always interested in my work, fueled my curiosity and inspired me to make new endeavors. Last, but not by no means least: Tobi. You have become part of my family shortly after we had met for the first time in Kumpula. I thank you for supporting me in so many ways. As a colleague you inspired me, helped me with sampling and lab work and gave me scientific advice. As my partner you always had open ears and arms for me when I needed support, but you also challenged the way I think, for which I am truly grateful. Tausend Dank! I måg di!

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I

Seasonal genotype dynamics of a Baltic dinoflagellate – pelagic populations are homogeneous and as diverse as benthic seed banks

Submission: Molecular Ecology

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Abstract

Genetic diversity is the basis for evolutionary adaptation and selection under changing environmental conditions. Phytoplankton populations are genotypically diverse, can become genetically differentiated within small spatiotemporal scales and many species form resting stages for seasonal survival. Resting stage accumulations (seed banks) are expected to serve as archives for genetic information, but so far, their role in phytoplankton diversity and evolution has remained unclear. In this study we used the toxic dinoflagellate *Alexandrium ostenfeldii* (Dinophyceae) as a model organism to investigate if (1) the benthic seed bank is more diverse than the pelagic bloom population and (2) if the pelagic population is seasonally differentiated. Cysts and bloom samples were collected in the Baltic Sea during 2015, followed by cell isolation and genotyping using microsatellites (MS) and restriction site associated DNA sequencing (RAD). High clonal diversity (98 – 100%) combined with intermediate to low gene diversity (0.58 – 0.02 depending on the marker) was found. Surprisingly, the benthic and pelagic fraction of the population were equally diverse, and the bloom was temporally homogenous, despite seasonal fluctuation of potential selection pressures. The persistence of linkage equilibrium throughout the year indicates frequent genetic recombination. Results of this study suggest that continuous benthic-pelagic coupling, combined with sexual reproduction prevent the establishment of a single clonal lineage in a dynamic environment. Together they harmonize the pelagic with the benthic population and thus prevent seasonal population differentiation. At the same time frequent sexual reproduction and benthic-pelagic coupling maintain high clonal diversity in both habitats.

1 Introduction

Phytoplankton evolution and their response to global change are of growing research interest due to their pivotal role in global nutrient and gas fluxes, as well as in aquatic food webs (Field et al., 1998). Because of increasing toxic bloom formation in coastal waters with potential effects on ecosystems, there is a rising demand for studies explaining the role of genetic diversity in relation to environmental change (Bach et al., 2018; Chust et al., 2017; Collins et al., 2013; Lassus et al., 2016; Rabalais et al., 2009). Within the past few decades our understanding of phytoplankton evolution has changed fundamentally from the assumption that marine phytoplankton populations are panmictic – due to argued high dispersibility and unlimited gene flow – to a more complex view of genetically differentiated populations. A growing body of literature suggests that phytoplankton populations can be temporally differentiated (Dia et al., 2014; Erdner et al., 2011; Gao et al., 2019; Godhe et al., 2016; Lebret et al., 2012; Richlen et al., 2012; Sassenhagen et al., 2018, 2015) and geographically structured due to physical barriers and / or environmental gradients (Godhe et al., 2016; Sjöqvist et al., 2015; Tahvanainen et al., 2012). Genetic structure developing within relatively short time scales or geographic distances implies that evolution may happen rapidly, e.g. within a few hundred generations (Collins et al., 2013). In order to become genetically differentiated, populations must be affected by one or several processes, like natural selection, mutation or genetic drift, in combination with limited gene flow (Rengefors et al., 2017).

Phytoplankton species are currently facing multifaceted and simultaneous climate-change-mediated alterations of their oceanic environment, in addition to existing natural environmental variability (Reusch and Boyd, 2013). These environmental changes require phytoplankton communities to respond by complex eco-evolutionary processes, including

short-term cellular acclimation, and long-term adaptation to selection based on standing genetic variation (Collins and Gardner, 2009). Genetic diversity is the basis for these processes and in the recent decade numerous studies evidenced that phytoplankton populations are genetically and phenotypically diverse (Rengefors et al., 2017). Diverse phytoplankton communities and populations are expected to contain a variety of phenotypic traits, facilitating successful adaptation to changing environmental conditions (Litchman et al., 2010) and resistance to perturbations (Ptacnik et al., 2008; Sjöqvist and Kremp, 2016). Recently the importance of large intraspecific diversity has been emphasized, potentially allowing rapid population level adaptation to climate change (Godhe and Rynearson, 2017; Wolf et al., 2019).

The relationship between diversity and evolution of phytoplankton communities is complicated by the ability of many taxa to form resting stages. Resting stages (or resting cysts) are cells with strongly reduced metabolic activity, formed by many phytoplankton groups to survive adverse environmental conditions (Fryxell, 1983). They may play different roles in the life cycle and are thought to be crucial for seasonal survival (Anderson and Rengefors, 2006; McQuoid and Hobson, 1996; Rengefors and Anderson, 1998; Reynolds, 1984). Furthermore, resting stages accumulating at the sea floor after consecutive growth periods can be considered archives of genetic information.

Seed banks are an important eco-evolutionary strategy, increasing the chance to persist through environmental variation in several ways: they facilitate increased genetic diversity and population differentiation in the presence of gene flow (Sundqvist et al., 2018), but can also conserve genetic structure of populations in the long run (Härnström et al., 2011; Ribeiro et al., 2011). Thus, seed banks may both slow down and enhance evolution by adaptation, depending on whether the fraction of emerging genotypes is a random or non-random sample

of the total gene pool (Hairston Jr and De Stasio Jr, 1988). The evolutionary role of seed banks also depends on the mode of reproduction and cyst formation. Sexual resting stage formation is generally considered to contribute to genetic diversity of eukaryotic phytoplankton populations (Rengefors et al., 2017; von Dassow and Montresor, 2011) and accumulation of sexually produced cysts could increase the genetic diversity of the seed bank, compared to a clonally growing seasonal pelagic population. To our knowledge, only one study has so far compared the diversity of a phytoplankton seed bank with the actively growing population and could not confirm a higher diversity in the seed bank of asexually produced resting stages of the diatom *Skeletonema marinoi* (Godhe and Härnström, 2010). Resting cyst production of dinoflagellates does not always require genetic recombination either, as demonstrated for some species (Figueroa et al., 2008; Jerney et al., 2019a; Kremp and Parrow, 2006). Thus, sedimentation of cysts produced without genetic recombination could in turn reduce the genetic diversity of the seed bank or balance it with a sexually reproducing pelagic part of the population.

Besides sexual reproduction several other mechanisms may sustain high genetic diversity of phytoplankton populations. Environmental heterogeneity can for example promote evolutionary diversification, even more with temporal fluctuation (Cooper and Lenski, 2010), which is a prevailing explanation for maintenance of genetic diversity (Godhe et al., 2016; Gsell et al., 2012). In seasonal regions phytoplankton blooms are subject to varying environmental factors throughout the year, potentially selecting for different geno- and phenotypes (Reusch and Boyd, 2013). Clonally reproducing genotypes or lineages, thriving under certain conditions, could outnumber less competitive genotypes and thus lead to seasonal genetic population structure. A large set of coexisting subpopulations was for example found in the globally abundant marine cyanobacterium *Prochlorococcus* and

differentiation was linked to seasonal selection (Kashtan et al., 2014). Alternatively, continuous germination of resting stages over time might result in a loss of differentiation and increase of gene diversity within a single clonally growing phytoplankton bloom (Lebret et al., 2012). Once successfully growing subpopulations form resting stages, they may increase the diversity of the seed bank.

Strong seasonality characterizes coastal waters of the Northern Baltic Sea, where high geno- and phenotypic diversity was reported for the dinoflagellate *Alexandrium ostenfeldii* (Paulsen) Balech and Tangen (1985), which grows from May to September and occasionally forms dense toxic blooms (Kremp et al., 2016; Tahvanainen et al., 2012). These are associated with local accumulations of benthic resting cysts (Hakanen et al., 2012; Kremp et al., 2009; Suikkanen et al., 2013), which makes *A. ostenfeldii* an excellent study organism to investigate if (1) the benthic seed bank is more diverse than the pelagic bloom population and (2) if the pelagic population is seasonally differentiated. Pre-requisites for increased seed bank diversity are clonal growth and genetic differentiation of subpopulations, without sexual recombination between them. Once selected genotypes of differentiated subpopulations form cysts, they can accumulate in the sediment and generate a seed bank with large diversity. If different subpopulations are part of the seed bank and germinate or become more abundant at different times of the season the pelagic population could be genetically structured. Based on these assumptions we hypothesize that: (1) The genetic diversity of the seed bank is larger, compared to the pelagic population and (2) the pelagic population is genetically differentiated during one season. To investigate *A. ostenfeldii* genotype dynamics and diversity a sampling campaign was carried out in 2015, followed by cell isolation and genotyping using microsatellites (MS) and restriction site associated DNA sequencing (RAD).

2 Material and Methods

Sampling

The sampling site (60°05, 6'N, 20°32, 4'E) is located in a shallow bay (water depth < 3 m) in the Föglö archipelago, Åland Islands, Northern Baltic Sea, which is an extensively studied bloom site of *A. ostenfeldii* (Hakanen et al., 2012; Jerney et al., 2019b; Kremp et al., 2009). The soft muddy bottom is partly densely vegetated and summer salinity ranges typically between 5 and 6 psu (practical salinity units). From December to April the bay is usually ice-covered and in summer water temperature can rise to +24 °C. Between March and October 2015 seven sampling campaigns were carried out (Table 1). At all dates water samples were taken to measure temperature, nutrients, Chl *a* level and to count *A. ostenfeldii* cells, as described in the following sections. At the end of March and in October sediment was sampled for isolation of *A. ostenfeldii* resting cysts (referred to as cyst populations C0 and C6, Table 1) and between June and August water samples were taken to isolate motile *A. ostenfeldii* cells (referred to as bloom populations B1 – B5). During the same period four replicate water samples were taken at each date to characterize algal toxins and the phyto- and zooplankton community (details below). Water chemistry, and phyto- and zooplankton community data were visualized with Sigma Plot V10.0 (Systat Software, San Jose, CA, USA).

Water chemistry

For nutrient analysis water samples (250 mL) were taken at 0.5 m water depth with a Ruttner sampler, transferred to hypochloric acid-washed 250 mL bottles and stored cool and dark until further processing. Nutrients were analyzed within 24 h, at the Marine Research Center of the Finnish Environment Institute (SYKE MRC) according to an in-house protocol (accredited by

the Finnish Accreditation Service). Total nutrients (TP and TN) and dissolved inorganic nutrients (NH_4, NO_3, NO_2, PO_4, SiO_4), were measured spectrophotometrically (Grasshoff et al., 2009; Koroleff, 1979). Temperature was measured in the field with a thermometer integrated in the Ruttner sampler and salinity was measured with a salinometer (Guildline's Autosal 8400B Laboratory Salinometer by OSIL) at SYKE MRC. Chlorophyll *a* (Chl *a*) was measured spectrophotometrically, according to SYKE MRC in-house protocol (HELCOM, 1988, 2017a).

For the sampling of hydrophilic paralytic shellfish poisoning (PSP) toxins, 10–20 L of seawater were filtered through a 100 µm net, onto a 25 µm sieve and material retained on the sieve was washed into a 50 mL centrifugation tube. The concentrated sample was filtered onto a Whatman GF/F glass fiber filter (Ø 25 mm), which was stored at -20 °C temporarily. For the analysis of PSP toxins, filters were placed in cryovials and approximately 0.9 g lysing matrix D was added to each vial followed by 1 mL 0.03 M acetic acid. Sample homogenization and toxin extraction was performed at maximum speed (6.5 m s^{-1}) for 45 seconds using a Bio101 FastPrep (Thermo Savant, Illkirch, France) instrument. Homogenized samples were centrifuged at 16,100 x g at 4 °C for 15 min, after which the supernatants were transferred to 0.45 µm pore size spin filters (Millipore Ultrafree, Eschborn, Germany) and centrifuged for 30 seconds at 6000 x g. Supernatants were transferred to HPLC vials for toxin analysis. PSP toxins were determined by ion pair liquid chromatography coupled to post-column derivatization and fluorescence detection, as described in Krock et al. (2007) and Van de Waal et al. (2015). Toxins PSTs (C1/2, dcGTX2/3, GTX2/3, GTX1/4, B1, dcSTX, STX, and NEO) were quantified by external calibration curves with standard solutions ranging from 5 pg μL^{-1} to 6 ng μL^{-1}. Detection limits for each toxin are given in Table S2.

Biotic environment

Water samples for phytoplankton counts were taken from 0.5 m depth with the Ruttner sampler and fixed with Lugol's solution. Depending on cell densities, sample volumes of 3 – 50 mL were settled in Utermöhl counting chambers for 3 – 24 h (Utermöhl, 1958) and counted at 200x and 400x magnification using an inverted microscope (Leica DMI3000 B). At least 500 cells were counted in total. Cell abundances were transformed into biomasses according to HELCOM (2017b). For isolation of *A. ostenfeldii* cells, plankton samples were collected with a 20 µm mesh plankton net and transferred to 200 mL culture flasks. Water samples (10 L) for Zooplankton analysis were taken from the surface with a measuring beaker, filtered through a 63 µm sieve, conserved in 70% EtOH and counted with an inverted microscope (Leica DMI LED) at 50x magnification according to HELCOM (2017c).

Isolation and culturing of *A. ostenfeldii*

For *A. ostenfeldii* cyst isolation four replicate sediment cores were sampled in April and October 2015. Sediment sampling and processing were carried out as described earlier in detail (Jerney et al., 2019b). Approximately 100 single resting cysts were isolated after both samplings and clonal cultures were established and maintained as explained previously (Jerney et al., 2019b, 2019a). Similarly, 84 -138 motile cells were isolated randomly from plankton samples after each sampling date from June to September. Clonal cultures were established as described above, but incubated at 20 °C (instead of 16 °C), 14:10 light:dark cycle and ~100 µmol photons m^{-2} s^{-1}.

Genotyping

2.1 Microsatellite markers (MS)

50 exponentially growing clones were randomly selected from each sampling date, 0.5 to 10 mL of each culture harvested and centrifuged at 10.000 rpm for 1 min. The obtained cell pellet was stored at -20 °C until further processing and DNA was extracted in 5% Chelex solution as described by Nagai et al. (2012). Primer pairs and the PCR conditions were described by Nagai et al. (2014) and all primer pairs, except of one for locus *Aosten144* were used in this study. PCR products were electrophoresed on an ABI 3730xl DNA Analyzer (Applied Biosystems) and allele sizes were determined using a 600 LIZ size standard (Applied Biosystems) and GeneMapper v 4.0 (ABI). Allele numbers at the 10 loci ranged from 2 to 12 (average of 5.3), and estimates of gene diversity (Nei, 1978) from 0.10 to 0.92, suggesting that the microsatellites are suitable to characterize genetic structure of *A. ostenfeldii* at the population level (Sildever et al., 2019).

2.2 Restriction site associated DNA sequencing (RAD)

Four sampling dates were included for RAD (C0, B1, B3, B5; Table 2) with 19 to 20 isolates per date. The cyst sampling, and three bloom samplings with maximal temporal distance were included to compare cysts with bloom samples and to increase the chance of capturing emerging population structure during the season. Exponentially growing cultures (approximately 10,000 cells mL^{-1}) were harvested and concentrated by centrifugation. After addition of 100 µL CTAB buffer the cell pellets were stored at -80 °C. A freeze-thaw cycle was repeated 3 times to break down cell walls and ease DNA extraction: samples were thawed at 65 °C for 5 min with a bench heating block and immediately frozen again at -80 °C for 24 h before starting the next cycle. DNA of each strain was extracted using a

cetyltrimethylammonium (CTAB)-based protocol (Dempster et al., 1999) modified as described by Sassenhagen et al. (2015).

2.3 RAD library preparations

A pilot test was first carried out with three strains (AOB325, AOB413, AOB504). From this data set a minimum number of reads and number of samples that can be multiplexed was determined. Genomic DNA was digested with high-fidelity SbfI (New England Biolabs), applying a RAD library preparation protocol modified form Amores et al. (2011) and Etter et al. (2011). Modifications (according to Rengefors et al, subm) included an increased amount of ligase (2000 U/µL T4 ligase) and decreased volume of NEB2 buffer (1 µL). In addition, AMPure XP beads were used to remove redundant P1 adapters and elution was done in three steps to increase the DNA yield, following the repair end and overhang addition step. AMPure XP beads were also used instead of column purification after the P2 adapter ligation. The final full amplification was performed with 16 µL template and 16 PCR cycles. After P1 adapter ligation 20 uniquely barcoded (6 bp) strains were multiplexed per lane. Samples were subsequently sequenced on a HiSeq2500 system (Illumina), using paired-end 125 bp read length and v4 sequencing chemistry.

2.4 RAD – single nucleotide polymorphism (SNP) identification

Quality control was done with FastQC version 0.11.6 (Babraham Bioinformatics), followed by de-multiplexing, and processing with Stacks software (Catchen et al., 2013, 2011) version 2.41. The Stacks pipeline was run manually. Prior to the main analysis, parameter settings were tested with a subset of 12 strains (3 from each population) with high coverage, as suggested by Paris et al. (2017) and Rochette and Catchen (2017). Two samples (B376 and C115) with less than one million reads were excluded from the final analysis. Overall the average coverage was very low (< 20x) despite optimized parameter settings. Therefore, the

parameter settings were selected to maintain a mean coverage of at least 10x to maximize the number of utilized reads and polymorphic SNPs, by varying mismatch (M) and depth of stack (m). The final Stacks parameters were set to -m 5 -M 1 (ustacks) and all other parameters were set to default. Raw counts and coverage were tracked at all possible stages (after process_radtags, ustacks, gstackst and populations). To investigate the population genetic structure and metrics the data were filtered with Stacks populations, following two approaches: to compare the diversity of the cyst population with the pelagic population, all bloom individuals were pooled and SNP loci found in two populations (cysts and pooled bloom population) and in at least 80 % of the individuals were considered (2p). To investigate seasonal differentiation SNP loci found in all four temporal subpopulations (C0, B1, B3 and B5) and in at least 80 % of the individuals were utilized (4p). Only the first SNP of each locus was included, and all data files were created directly with Stacks populations for downstream analyses. Potential human or bacterial contaminant sequences were identified using the taxonomic sequence classifier Kraken2 (Wood et al., 2019).

Population genetic metrics and statistical analysis

Genetic diversity (π) for RAD data was calculated directly in Stacks based on all SNPs utilized by the Stacks population program. Data on number of RAD sites, variant alleles, and polymorphic sites were also obtained. Data exploration and all other statistical analysis for RAD and MS data were carried out with R version 3.6.1 (R Core Team, 2018) and R studio version 1.2.5019 (RStudio Team, 2019) focusing on analysis suitable for non-model populations including clonal or partially clonal organisms (Grünwald et al., 2017).

Basic population metrics of MS data were extracted using the package poppr (Kamvar et al., 2015, 2014). Genotypes with more than 25 % missing data were excluded from the statistical analysis of both datasets, population differentiation (F_{st}) was calculated in R, package

hierfstat (Goudet and Jombart, 2015; Nei, 1973), and p-values were obtained after 999 permutations followed by a Monte Carlo Test (package ape4, Bougeard and Dray 2018). In addition pairwise genetic distances D (Jost) and Gst (Hedrick) were calculated with package mmod (Winter, 2012). Allelic richness was calculated with the package PopGenReport (Adamack and Gruber, 2014). Analysis of molecular analysis (AMOVA) was performed with the help of package poppr (Kamvar et al., 2015, 2014) and followed by a Monte-Carlo test. Package ggplot2 (Wickham, 2016) was used for plotting the results and packages magrittr (Bache and Wickham, 2014) and dplyr (Wickham et al., 2019) were used for data manipulation. The mode of reproduction was assessed for both data sets by evaluating observed linkage among loci against expected distributions from permutation (999 permutations) using the index of association (I_A) described by Brown et al. (1980) and index $\bar{r}_d$, which accounts for the number of loci sampled and is less biased (Agapow and Burt, 2001). I_A and $\bar{r}_d$ were assessed with the package poppr (Kamvar et al., 2015, 2014).

Population structure was investigated using principal components analysis (PCA) in R with package adegenet (Jombart and Ahmed, 2011) and remaining missing values were ignored. In addition, a k-means clustering approach, which identifies clusters using successive k-means, was used from the same package. These functions implement a clustering procedure of running successive k-means with an increasing number of clusters (k), after transforming data using a PCA. For each model, a statistical measure of goodness of fit (Bayesian information criterion = BIC) was computed to choose the optimal k. Furthermore, a discriminant analysis of principal components (DAPC) was carried out, followed by DAPC-cross validation to choose the number of principal components retained.

3 Results

Sequencing and basic metrics

Between 84 – 138 single cells were isolated after each sampling, of which 28 – 50 % were culturable (Table 1). With microsatellite markers 261 strains were successfully characterized in total, based on 9 loci and 54 alleles. A genotype accumulation curve showed that at least 9 loci are required to discriminate between unique individuals. Four genotypes with more than 25 % missing values were excluded from the MS analysis. Of the remaining 257 genotypes the number of multilocus genotypes (MLG) was between 24 (B2) and 45 (C0 and B1) and 4 clones were detected (Table 1). Reads of the RAD dataset were 113 bp in length, the mean number of RAD reads per sample was 17,032,952 (± 839,864 SE) and 5,884,607 loci (i.e.86 % of the reads) were assembled into a contig. The effective per-sample mean coverage was 26.8 x. After filtering SNP loci found in at least 80 % of the individuals and the two subpopulations (2p), 415 polymorphic RAD loci remained (830 alleles). After filtering SNP loci occurring in at least 80 % of the individuals and all four temporal subpopulations (4p), 246 polymorphic RAD loci remained (492 alleles). The mean length of loci was 585.98 bp (± 3.72 SE) and 587.21 bp (± 2.82 SE) for the 4p and 2p filtering approach. Nine genotypes from the 2p and two genotypes from 4p dataset were excluded as they had more than 25% missing values. All genotyped individuals used for RAD were distinct. Sequence classification of the resulting loci using Kraken 2 identified 17.16 % (SD ± 7.74) of sequences as of potential bacterial origin and 4.78 % (SD ± 1.78) of sequences as of potential human origin.

Table 1. Culture establishment and population metrics of microsatellite data. C refers to cyst populations, B refers to bloom populations, numbers indicate different time points; MLG = number of multilocus genotypes; He = Nei's (1978) gene diversity; 1-D = Simpson index of diversity; I_A = index of association; p-value obtained after 999 permutations; $\bar{r}_d$ = I_A adjusted for the number of loci; p-value obtained after 999 permutations, I_A and $\bar{r}_d$ calculations were based on randomly subsampled populations (n = 20).

Sampling date	Population	Cells isolated (n)	Cultures established (%)	Genotyped isolates (n)	MLG	He	1-D	I_A	$\bar{r}_d$	mean allelic richness
31-Mar	C 0	100	50	46	45	0.58	0.98	-0.11	-0.01	4.01
02-Jun	B 1	132	37	45	45	0.54	0.98	0.05	0.01	3.85
30-Jun	B 2	84	35	24	24	0.55	0.96	0.11	0.01	4.15
26-Jul	B 3	138	36	44	44	0.56	0.98	0.07	0.01	3.59
11-Aug	B 4	92	45	36	34	0.53	0.97	0.07	0.01	3.64
25-Aug	B 5	108	28	29	29	0.53	0.97	-0.17	-0.02	3.81
19-Sep	C 6	100	34	33	32	0.53	0.97	-0.11	-0.01	3.75

Diversity measures

High genotype (or clonal) diversity, which is the ratio of the number of unique genotypes (G) to the total number of isolates genotyped (N), was calculated with MS data (G:N = 0.98) and RAD data (G:N = 1.00). Gene diversity (Nei, 1978) varied from ~ 0.53 (B4) to 0.58 (C0) based on MS data. The number of alleles per locus ranged from 3 to 9 with an average of 5.4 (Table S1) and the mean allelic richness after rarefaction was between 3.64 (B3) and 4.15 (B2) (Table1). Both RAD data sets (2p and 4p) had a mean gene diversity of 0.02 - 0.03, which equals the nucleotide diversity (Pi, Table 2), as we only utilized the first SNP of each locus. Random subsampling (n = 18) of the 2p data set did not alter the gene diversity. Allelic richness ranged from 1.16 (C0, C) to 1.20 (B5, B) for both RAD datasets. The highest number of private alleles occurred in the B5 (58) and the lowest number in the B3 (42) population. The level of polymorphism was generally very low, based on RAD data 0.05 % of all sites were polymorphic.

Table 2. Basic population metrics of RAD-loci, filtered to include at least 80% of the individuals in either 4 subpopulations (C0, B1, B3, B5) or 2 subpopulations (B=bloom, C=cyst). N = number genotyped isolates. π = estimate of nucleotide diversity of variant sites; I_A = index of association; p-value obtained after 999 permutations; $\bar{r}d$ =I_A adjusted for the number of loci; p-value obtained after 999 permutations; I_A, $\bar{r}d$ and allelic richness were calculated based on rarefaction analysis (n = 19 for C0, B1, B3, B5 and n = 18 for C and B). Bold numbers indicate significant deviation from a random distribution (p < 0.05).

Population	N	Private alleles (n)	Polymorphic loci (%)	π	I_A	$\bar{r}d$	mean allelic richness
C	19	76	0.05	0.02	0.40	0.00	1.16
B	59	292	0.13	0.03	-0.03	0.00	1.20
C 0	19	46	0.05	0.02	-0.05	0.00	1.16
B 1	20	50	0.05	0.03	-0.08	0.00	1.18
B 3	19	42	0.05	0.02	**0.51**	**-0.01**	1.17
B 5	20	58	0.06	0.03	0.01	0.00	1.20

Table 3. Pairwise genetic distances. B = Bloom, C = cyst populations: F_{st} for msat data below the diagonal, and RAD data above the diagonal (Nei, 1973) p-values obtained with a Monte-Carlo test after 999 permutations indicated no significant difference for all pairwise comparisons).

	C 0	B 1	B 2	B 3	B 4	B 5
C 0		0.021		0.020		0.022
B 1	0.010			0.020		0.016
B 2	0.014	0.015				
B 3	0.015	0.025	0.017			0.019
B 4	0.016	0.023	0.019	0.014		
B 5	0.016	0.016	0.027	0.017	0.013	
C 6	0.018	0.017	0.015	0.018	0.009	0.012

Table 4 Pairwise genetic distances for msat and RAD data: D (Jost) above / Gst (Hedrick) below the diagonals.

msat								**RAD**				
	C0	**B1**	**B2**	**B3**	**B4**	**B5**	**C6**		**C0**	**B1**	**B3**	**B5**
C0		0.010	0.022	0.023	0.026	0.023	0.029	**C0**		0.023	0.023	0.023
B1	0.004		0.016	0.041	0.037	0.021	0.024	**B1**	-0.008		0.023	0.024
B2	0.009	0.007		0.025	0.024	0.039	0.013					
B3	0.009	0.017	0.010		0.019	0.023	0.026	**B3**	-0.008	-0.008		0.023
B4	0.011	0.017	0.010	0.008		0.012	0.003					
B5	0.010	0.009	0.017	0.010	0.005		0.008	**B5**	- 0.008	-0.008	-0.008	
C6	0.012	0.011	0.006	0.011	0.002	0.004						

Linkage equilibrium

Based on MS data the index of association (I_A) and the index of association adjusted for the number of loci ($\bar{r}_d$) of each temporal subpopulation ranged from -0.17 to 0.11 and -0.02 to 0.01 (Table 1). P-values obtained for both indices indicated no significance. For the 2p RAD data set I_A values of 0.4 (C) and -0.03 (B), corresponding to $\bar{r}_d$ values of 0.00 were calculated (Table 2). For the 4p RAD dataset the I_A was between -0.08 (B1) and 0.51 (B3), corresponding to $\bar{r}_d$ = 0.00 (B1) and -0.01 (B3). The I_A and $\bar{r}_d$ obtained for B3 were significantly different from a random distribution ($p < 0.05$, Table 2, Figure 1).

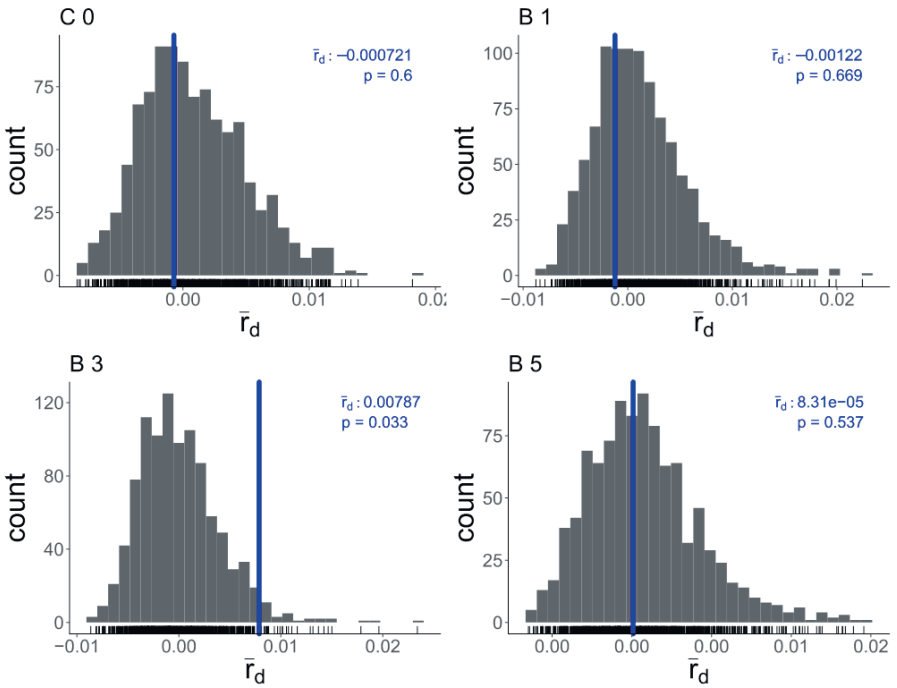

Figure 1. Indices of association adjusted for the number of loci ($\bar{r}_d$) of each temporal subpopulation, based on RAD data are indicated by the blue line (p-value obtained after 999 permutations, n = 19). The grey area represents the re-sampled distribution of unlinked loci. Based on $\bar{r}_d$ there is no evidence of linkage disequilibrium among loci for C0, B1 and B5 (P>0.25), consistent with a sexually recombining population. Only for B3 the hypothesis of no linkage among markers is rejected (P<0.05), indicating a higher degree of clonality.

Genetic differentiation and population metrics

The analysis of microsatellite and SNP data showed that there was weak, non-significant differentiation between all population pairs (Table 3 and 4). Pairwise F_{st} values were between 0.009 (B4-C6) and 0.027 (B2-B5) for the MS data and between 0.016 (B1-B5) to 0.022 (C0-B5) for the 4p RAD data (Table 3). Pairwise genetic distances between the cyst population sampled in spring (C0), and each of the temporal pelagic populations increased marginally towards the end of the season (0.010 – 0.018, B1 to C6), but there was no significant differentiation. The pairwise distances were higher at B1-B3 (0.025) and B1-B4 (0.023), but none of the F_{st} values were significant. When comparing the pooled bloom isolates with the cyst isolates the F_{st} value was even lower after rarefaction (0.011). D (Jost) values ranged from 0.002 (C6-B4) to 0.017 (B1-B3 and B2-B5) based on MS data and displayed no variation for the 4p RAD data (-0.008 between all pairs). Pairwise Gst (Hedrick) ranged from 0.003 (B4-C6) to 0.041 (B1-B3) for the MS data and was 0.023 between all population pairs based on 4p RAD data, except for B1-B5 (0.024). The analysis of molecular variance (AMOVA) results indicated no population differentiation across the season and between the benthic and pelagic population (Phi = -0.003). 100% variation was found at the population level, with no significant differences between populations (P = 0.694 for p4 and P = 0.625 for p2, Table S3).

Population genetic structure

Principal components analysis (PCA) showed that the first three principal components explained less than 15 % of the total variance in the MS and the 4p RAD dataset. With increasing number of principal components eigenvalues decreased gradually (without a sharp decrease). Thus, plotting the first two principal components was not meaningful to display the variation of the datasets and PCA plots are not shown. A K-means cluster analysis, based on allele frequencies, showed that there was no support for more than one cluster (K = 1) based

on Bayesian Information Criterion (BIC). BIC values increased steadily with the number of clusters, indicating that one cluster explained the variation of the dataset best. Posterior membership probability plots, based on a discriminant analysis of principal components (DAPC), showed that all individuals belong to one group, therefore no plots are presented. DAPC cross-validation indicated that the proportion of successful outcome prediction reached from 0 to 60% for any number of principal components retained (1-78) but was highest for 47 principal components (90%).

Selection regimes

Abiotic and biotic environmental data displayed a pronounced seasonal variation (Figure 2). The environmental conditions at the first cyst sampling C0 (end of March) was characterized by a low temperature (3°C) and low nutrient levels (TN and TP below 1 µmol L^{-1}), combined with a low Chl *a* level (11.6 µg L^{-1}), and no *A. ostenfeldii* cells in the water column (Figure 2d). In addition, the highest inorganic N:P ratio (64) of the season was detected. In the beginning of June (B1) the temperature had increased to 14.4 °C and a low number of *A. ostenfeldii* (360 cells L^{-1}) was found together with high total toxin cell quota of 8.3 pg $cell^{-1}$. At the same time a low phytoplankton biomass (~ 1 mg L^{-1}, Figure 2a), a high zooplankton biomass (1800 individuals L^{-1}, Figure 2c) and elevated nutrient concentrations (TN and TP, Figure 2e) were measured. A different situation was observed at the end of June (B2), when the temperature had reached 20 °C. An increase of the *A. ostenfeldii* abundance (~ 1000 cells L^{-1}) and toxin cell quota (12.0 pg PSP toxins $cell^{-1}$) co-occurred with the highest zooplankton abundance (2300 individuals L^{-1}), but the overall phytoplankton biomass and Chl *a* remained low. A prolonged period of elevated temperatures (above 15 °C) occurred in July and August (B3 – B5), with decreasing zooplankton abundance (below 360 individuals L^{-1}) and increased growth of *A. ostenfeldii* (above 6000 cells L^{-1}, Figure 2d) and other phytoplankton (in total 14

mg L^{-1}, Figure 2a), corresponding to a Chl *a* peak of ~110 µg L^{-1} (B5). During this period, we measured the second highest total toxin cell quota of the season (8.9 pg $cell^{-1}$, B4 sampling, Figure 2b) and a maximum concentration of TN and TP (~ 80 and 3 µmol L^{-1}, Figure 2e). During the B5 sampling the ratio of inorganic N:P was ~50, whereas N:P ratios were below 5 during all other bloom samplings. Towards the end of the season (sampling C6 in September) temperature and the Chl *a* level had declined to 16 °C and 65 µg L^{-1}, but total phytoplankton biomass was still high (~12 mg L^{-1}) and the cell density of *A. ostenfeldii* reached its maximum of 11,844 cells L^{-1}.

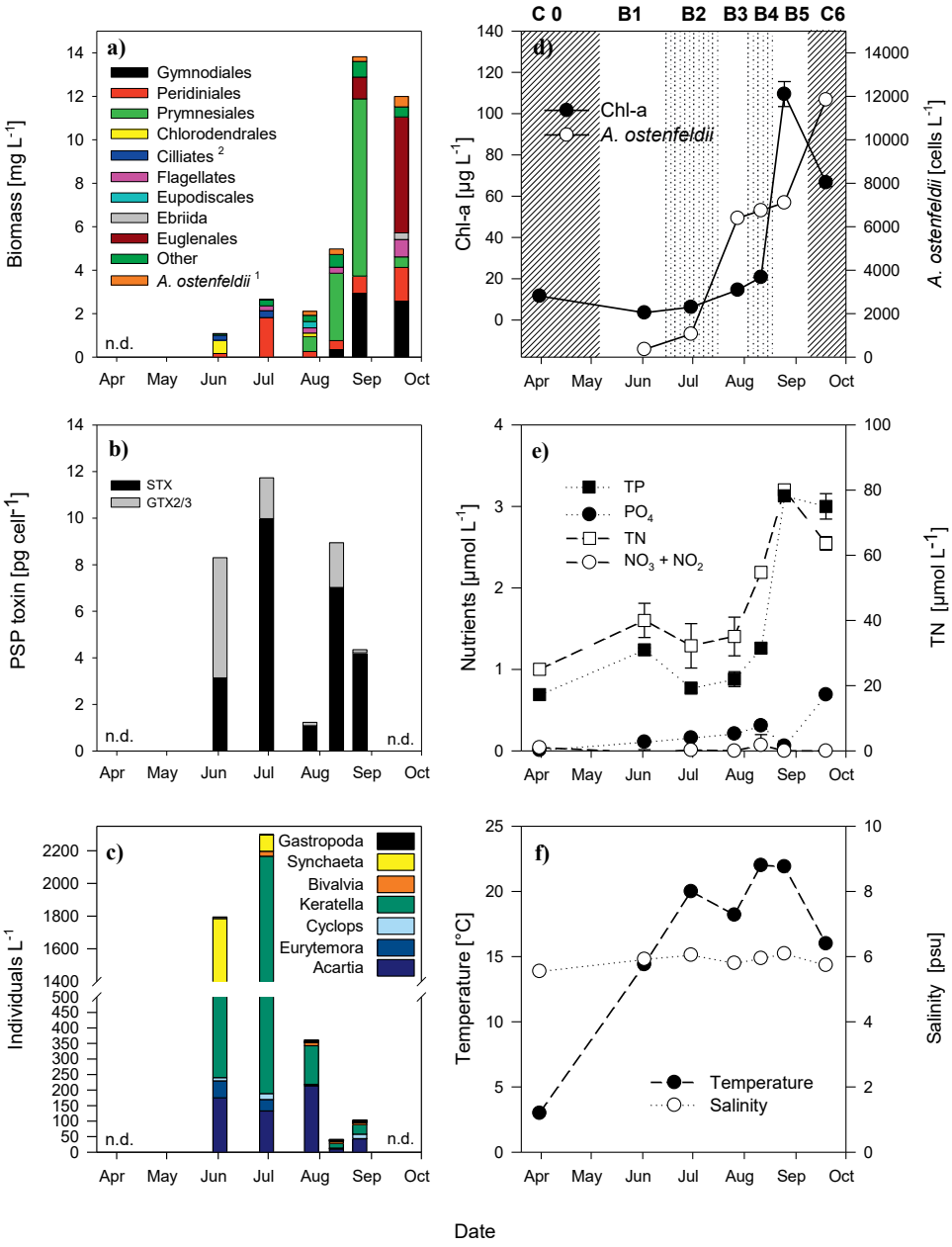

Figure 2. Biotic and abiotic environmental parameters (mean values, n=4). a) Phytoplankton taxa contributing to more than 80% of the total biomass, [1] Gonyaulacaceae, [2] with endosymbiotic algae, b) A. ostenfeldii toxins, c) Zooplankton community, d) Total Chl a level and cell concentration of A. ostenfeldii, e) Nutrient concentrations, f) Water temperature and salinity; n.d. = not determined.

4 Discussion

Algal blooms can have a duration of several weeks to months, despite short generation times and substantial changes in the aquatic environment during the bloom period. Consequently, it has been suggested that the blooms are exposed to shifting selection regimes, which could result in separate sub-populations that are favored at different times during the growth season. Here, we tested the hypotheses that a bloom consists of separate subpopulations, and that the cyst “seed bank” serves as a genetic reservoir with high genetic diversity. Contrary to our expectations, we found little evidence of population differentiation within a bloom of the dinoflagellate *Alexandrium ostenfeldii*. Moreover, genetic diversity was equally high in bloom and cyst populations.

Seed bank diversity – unravelling the reservoir function

Both types of genetic marker applied in this study revealed similar diversity levels in the benthic and the pelagic phase of a Baltic *A. ostenfeldii* population, which contrasts with the common expectation of increased diversity of seed banks (Rengefors et al., 2017) or zooplankton egg banks (Brendonck and De Meester, 2003). Circumstances that would facilitate an increased diversity in the seed bank, compared to the actively growing part of the population in the water column, are accumulation of sexually produced cysts in the sediment, combined with germination and growth of a smaller subset of the entire population, and limited exchange between the benthic and pelagic habitat. For most dinoflagellates with a known life cycle, resting cysts are a result of sexual reproduction, whereas asexual proliferation and selection occur in the pelagic phase of the life cycle (Rengefors et al., 2017). Thus, a “typical” dinoflagellate life cycle could promote increased diversity of the benthic seed bank, compared to a mainly clonally growing subset of the pelagic population, if genotypes with certain traits are selected and germinate. On the other hand, successive recruitment of

diverse genotypes has been considered as a factor explaining high genetic diversity in prolonged phytoplankton blooms (Lebret et al., 2012). We can assume that the shallow water at the Föglö sampling site facilitates constant exchange between the seed bank and the plankton through encystment and germination, as depicted in Figure 3. *A. ostenfeldii* has a generalist life cycle strategy and forms diverse cyst types, including sexual heterothallic and asexual (and/or sexual homothallic) ones, under a variety of conditions (Jerney et al., 2019a, 2019b) and can germinate within a wide temperature window throughout the season (Figueroa et al., 2008; Jerney et al., 2019a). Frequent cyst formation and germination throughout the season would ensure strong coupling between the seed bank and the pelagic part of the population, which will harmonize the diversity between the two phases.

Our results for clonal diversity (0.97 MS and 1.00 RAD) and gene diversity *He* (0.53 to 0.58 MS) fall within the range of other studies on *Alexandrium*, reporting clonal diversity of 0.47 to 1.0 and gene diversity of 0.54 to 0.88 (Collins et al. 2013 and references herein). Nagai et al. (2004) reported a gene diversity of up to 0.97 for *A. catenella* (reported as *A. tamarense*), but relatively low gene diversity of 0.27 were reported for *A. tamiyavanichii* (Nishitani et al., 2009). RAD data presented in this study suggest a much lower gene diversity (equal to π, between 0.02 to 0.03), compared to MS data and previously reported values (0.07 to 0.11), derived from Amplified Fragment Length Polymorphism (AFLP) markers (Tahvanainen et al., 2012). Likewise, different markers revealed diverging results for *A. catenella* (reported as *A. tamarense*): Gene diversity of North Sea populations ranged from 0.03 to 0.51 based on AFLP loci and from 0.31 to 0.83 based on microsatellite loci (Alpermann et al., 2010). Inconsistent gene diversity results obtained with different markers for the same species most likely reflect marker specific characteristics (Fischer et al., 2017), as discussed later.

Frequency of sexual reproduction

To determine if populations are in linkage equilibrium or not, the linkage between loci was tested by calculating the index of association (I_A) and the index of association, adjusted for the number of loci sampled ($\bar{r}_d$) (Agapow and Burt, 2001). For clonal growth significant disequilibrium is expected due to linkage among loci, whereas for sexual growth linkage among loci is not expected. The null hypothesis tested is that alleles observed at different loci are not linked if populations are sexual, while alleles recombine freely into new genotypes during the process of sexual reproduction. Values close to zero indicate linkage equilibrium, while deviations from zero indicate disequilibrium. In this study low and non-significant $\bar{r}_d$ values were calculated for most of the season. Thus, we fail to reject the null hypothesis of no linkage among markers and conclude that sexual reproduction occurs not only at the end of the growth season, but more frequently in the pelagic phase. Only subpopulation B3 exhibited weak significant linkage disequilibrium (LD) based on RAD data (Table 2, Figure 3), pointing at increased clonal reproduction at the end of July. During this time high clonal growth and increased intra- and interspecific competition, could have selected for the most competitive clonal lineages. Weak, but significant LD has been reported earlier for other Baltic *A. ostenfeldii* populations, except of the Föglö population (Tahvanainen et al., 2012), which is in line with results of this study. Other studies that have addressed reproduction patterns in population genetic analyses, reported weak or no LD (Alpermann et al., 2009; Dia et al., 2014; Sassenhagen et al., 2015; Wyngaert et al., 2015), indicating that regular recombination events occur in phytoplankton populations despite their primarily asexual mode of growth. Sexuality indicators may be present in some and absent in other populations or subpopulations (Tahvanainen et al., 2012; Wyngaert et al., 2015), which suggests that the level of sexual reproduction, and consequently genetic diversity, is influenced by e.g. habitat

conditions, life-cycle strategies or population history (Rengefors et al., 2017). The frequency of sexual reproduction has a strong impact on short term evolution, as it may erase genetic structure emerging during a bloom event (Dia et al., 2014).

Seasonal population structure and selection

Pairwise genetic distances calculated for MS and RAD data (Table 3 and 4) suggest that the *A. ostenfeldii* bloom populations were hardly differentiated throughout the entire bloom season. There was a weak trend suggesting diversification, indicated by increasing pairwise genetic distances between C0 and B1-B5, but this trend was not significant (MS, Table 3 and 4). No significant population structure or variation of diversity was detected, despite seasonal variation of potential selection pressures (Figure 3). For populations to become genetically differentiated, one or more of the following processes are involved: natural selection, mutation or genetic drift, in combination with limited gene flow (Rengefors et al., 2017). Some likely explanations for the lack of genetically differentiated subpopulations are the following; First, no single clone dominates for long enough to form a separate subpopulation. This can be due to frequent sexual reproduction and resulting recombination, as indicated by the index of association. It could also be explained by a dynamic environment that never allows dominance of one or a few clones, analogous to the Intermediate Disturbance Hypothesis (Connell, 1978). Secondly, population differentiation may be prevented by high plasticity, which counteracts selection for different ecotypes/subpopulations.

The investigated *A. ostenfeldii* bloom site represents a dynamic environment with seasonal variation of potential selection pressures. It can be assumed that high predation and competition for nutrients represented selection pressures at the end of July and in August, indicated by peaks in zooplankton (B2) and phytoplankton abundance (B5). During the B5 sampling the inorganic N:P ratio of ~50, which is above 16 and suggests phosphorus

limitation based on Redfield (1958). In comparison, inorganic N:P ratios were below 5 during the other bloom samplings, pointing at nitrogen limitation during the rest of the season (Redfield, 1958). Allelic richness estimates derived from rarefaction analysis showed that B4 had the lowest diversity (Table 1). During this sampling date the second highest PSP toxin cell quota were measured (equaling highest toxin concentration per L in the water, Figure S1), suggesting that clonal lineages with similar alleles and traits were more frequent.

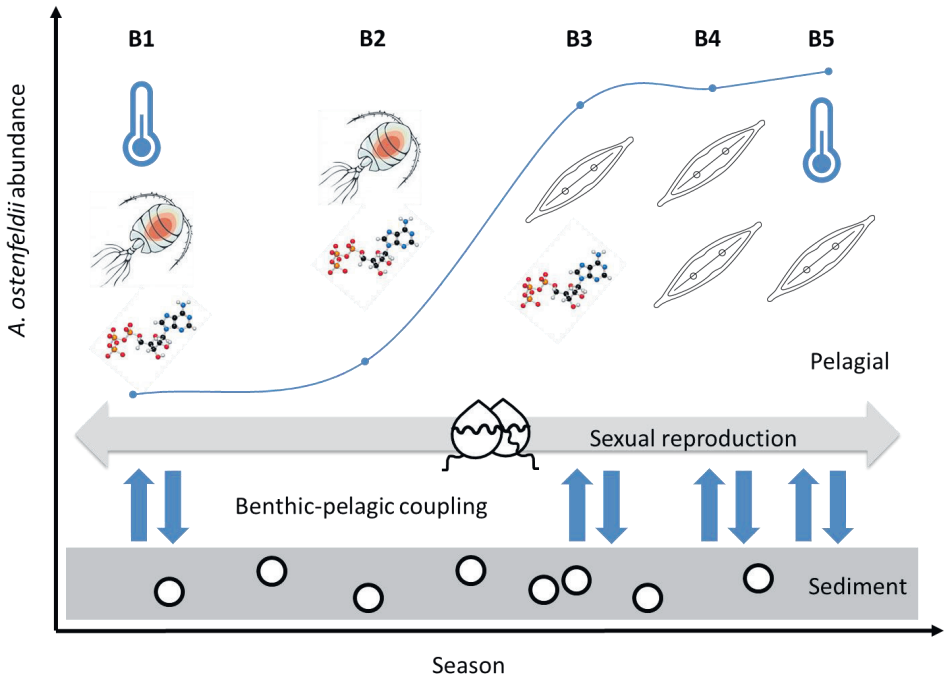

Figure 3. Graphic scheme, illustrating potential selection pressures and antagonistic processes (sexual reproduction and benthic-pelagic coupling) (sampling dates B1-B5). Selection pressures are indicated by symbols: Thermometers represents low temperature, copepods indicate grazing pressure, molecules stand for nutrient limitation and the diatom frustule for competition. A. ostenfeldii abundance is indicated by a blue line and dots.

Previous studies on other *Alexandrium* species have reported temporal (Alpermann et al., 2009; Richlen et al., 2012) and spatiotemporal (Dia et al., 2014; Gao et al., 2019) genetic intra-bloom differentiation. These were likely driven by natural selection through

environmental conditions, such as nutrient concentrations (Gao et al., 2019), but in the diatom *S. marinoi,* the presence of linkage disequilibrium and occasional lower allelic richness and reduced gene diversity in the pelagic populations, was interpreted as a result of stronger selection in the plankton populations (Godhe and Härnström 2010). Nevertheless, a homogeneous long-term population structure has been maintained in this population of *S. marinoi* over thousands of generations, despite weak genetic differentiation in the pelagic phase (Härnström et al. 2011). Another diatom study reported significant progressive loss of genetic differentiation during an arctic bloom within a few weeks, coupled with stable genetic diversity levels, but predominantly asexual growth is assumed for this species and resting stages are not known (Tammilehto et al., 2016).

MS vs RAD

Genome-wide analyses of SNP diversity were suggested to be more powerful, compared to MS markers to study genetic diversity within and among populations, because a limited number of microsatellite markers, marker ascertainment bias, and a high variance in microsatellite-derived diversity estimates may not adequately reflect genome-wide genetic diversity (Fischer et al., 2017). MS mutation rates are orders of magnitude higher and much more variable than those of single SNPs and almost certainly do not reflect those of the genome as a whole (Hodel et al., 2017). Thus, the unusually high mutational properties, which makes MS excellent for distinguishing different individuals, may inflate statistics such as F_{st} relative to the rest of the genome (Hodel et al., 2017). F_{st} values calculated for both markers were similar in this study, although slightly higher based on the RAD data. Since F_{st}'s are typically underestimated when based on microsatellites, the SNP based F_{st} is likely more reliable. Estimates of F_{st} may become inflated as missing data increase (Hodel et al., 2017), thus strict filtering (SNPs present in 80 % of individuals and exclusion of genotypes with more

than 25 % missing data) was applied in this study to avoid F_{st} inflation. However, a more liberal filtering of loci could retain loci valuable for population differentiation inference, since inflation of population genetic statistics might not happen as quickly as implied in simulation studies when loci with missing data are added (Hodel et al., 2017). Thus, more information about population structure might have been lost due to the strict filtering of loci applied in this study. Furthermore, the low SNP-based gene diversity of the present study might be an underestimation potentially arising from a bias in allele frequency estimation due to non-random haplotype sampling when using restriction digestion (Arnold et al., 2013). This could be because some recognition sequences themselves will be polymorphic, resulting in missing data for some chromosomes, and nonrandom sampling of lineages in a sample (Arnold et al., 2013). Additionally a high degree of repetitive sequences characterizing the *A. ostenfeldii* genome (Jaeckisch et al., 2011) could contribute to the relatively low RAD diversity estimates. The vast majority of the *A. ostenfeldii* genome appears to consist of large tandem arrays, and together they comprise at least 58% of the total sequence (Jaeckisch et al., 2011), which may be captured with RAD, but not with highly variable MS. An additional issue could be the size of the *A. ostenfeldii* genome. Because the genome is very large (115 pg $cell^{-1}$, Figueroa et al., 2010) many loci that are unique were maybe not covered in the analyses because they were excluded by the strict filtering settings. Thus, it is possible that preferentially repeated or conservative regions were included in the analysis leading to an underestimation of gene diversity and F_{st}. Despite minor deviations, results of both markers are in line in this study and highlight the importance of comparing alternative genotyping methods to study non-model organisms.

Limitations of this study

The relatively low culture establishment success from benthic and pelagic samples in this study (28-50%, Table 1), means that 50% or more of the genetic diversity and variation were potentially not captured. This limitation is common for many culture-based genotyping studies, but culturing success is rarely reported. The survival rate in a previous *A. ostenfeldii* study ranged from 35 to 71% in different Baltic populations (mean survival rate was 51.6 %) (Tahvanainen et al., 2012) and is similar to the survival rate of e.g. *A. minutum* (42.7 %) (Dia et al., 2014) or *A. fundyense* (38 %), (Erdner et al., 2011) and this study.

In addition, the relatively high classification of sequences as bacterial, compared to other phytoplankton, e.g. 0.1 % for the raphidophyte *Gonyostomum semen* (Rengefors, Gollnisch, Ahrén, submitted) and around 3% for the dinoflagellates *Apocalathium* sp. (Rengefors, Ahrén, Wallenius, Annenkova, Kremp, unpubl.) might have blurred signs of seasonal population structure. At the same time, the relevance of the bacterial community for this species' metabolism is emphasized, since associations between the microbiome and *A. ostenfeldii* were suggested to be of importance for the dinoflagellate metabolism at brackish conditions (Sörenson et al., 2019).

Evolution and adaptation

It can be assumed that the seed bank slows down *A. ostenfeldii* evolution if a random set of resting stages with a large pheno- and genotypic diversity germinates but remains unaffected by natural selection. This could be facilitated by high plasticity, which can weaken the effect of natural selection on individuals and their associate traits (Chevin et al., 2010). High plasticity of growth rates was reported from transplant experiments with this species (Jerney et al., 2019b) and is supported by flexible cyst formation and germination (Jerney et al., 2019a).

Thus, the population is buffered against seasonal selection and rapid differentiation, which can slow down evolution. Simultaneously, the large clonal diversity can facilitate evolutionary adaptation if selection pressures last longer, become more intense or benthic-pelagic coupling is prevented or strongly reduced. Thus, global change related environmental variation will most likely not compromise the occurrence of this species in the Baltic Sea, as large phenotypic plasticity and a generalist life cycle were reported (Jerney et al., 2019b, 2019a), in addition to the high clonal diversity. Additionally, new genotypes with highly variable competitive abilities may evolve in timescales significantly shorter than climate change (Bach et al., 2018).

Conclusions

Similar diversity levels of the benthic and pelagic *A. ostenfeldii* population fractions and a lack of seasonal structure, despite pronounced selection pressures, most likely result from several processes. Tight benthic-pelagic coupling, combined with sexual and asexual growth and cyst formation, maintain gene flow throughout the season and harmonize the diversity in both zones. A lack of seasonal population structure could be associated with high plasticity, intermediate disturbance or too weak selection by environmental factors. In addition, sexual reproduction seems to be important for maintaining high clonal diversity of the local *A. ostenfeldii* population through the growth phase. Local seed banks provide a buffer against short term environmental variability and potentially slow down evolution. Simultaneously, long-term adaptation to future environmental conditions is possible because genetic diversity is stored, and new genotypes arise from frequent recombination.

Acknowledgements

We thank Johan Franzén and Tobias Lipsewers for assistance with sampling and field work. Marie Svensson, Ritsuko Kubota and Johanna Oja are acknowledged for their extensive support of lab work and culture maintenance. This work was funded in part by grants from the Academy of Finland (grant 310449 to SS and AK), Walter and Andrée de Nottbeck Foundation (JJ), and a Grant-in-Aid for Scientific Research (Kiban-B) by the Japan Society for the Promotion of Science (18KK0182) (SN). Sequencing was performed by the SNP&SEQ Technology Platform in Uppsala. The facility is part of the National Genomics Infrastructure (NGI) Sweden and Science for Life Laboratory. The SNP&SEQ Platform is also supported by the Swedish Research Council and the Knut and Alice Wallenberg Foundation. The authors wish to acknowledge CSC – IT Center for Science, Finland, for computational resources.

Conflict of interests

None declared.

Supplementary material

Table S1. Allele frequencies (alleles = Number of observed alleles, 1-D = Simpson index, He = Nei's (1978) gene diversity)

Locus	alleles	1-D	He	Evenness
Aosten10	3	0.10	0.10	0.41
Aosten13	6	0.74	0.75	0.78
Aosten101	9	0.73	0.74	0.71
Aosten126	5	0.62	0.62	0.83
Aosten171	3	0.54	0.54	0.83
Aosten296	4	0.50	0.50	0.78
Aosten359	5	0.72	0.72	0.90
Aosten436	9	0.55	0.55	0.50
Aosten685	5	0.45	0.45	0.60
mean	5.4	0.55	0.55	0.70

Table S2. Limits of detection (LOD) range for the individual PSP toxins during the entire season.

	LOD (pg cell^{-1})		
C1/2	0.05	-	1.86
GTX4	0.02	-	0.70
GTX1	0.02	-	0.75
dcGTX2	0.06	-	2.34
dcGTX3	0.46	-	17.49
GTX2	0.02	-	0.87
B1	0.03	-	1.03
GTX3	0.40	-	15.09
NEO	0.17	-	6.49
dcSTX	0.20	-	7.39

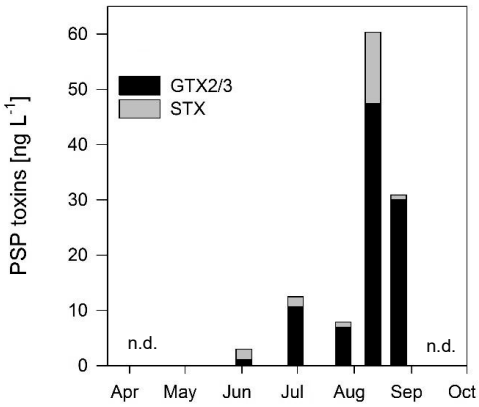

Figure S1. A. ostenfeldii toxins contained in one L of seawater.

Table S3. Analysis of molecular variance (AMOVA) summary of the genetic variation in the two RAD-seq datasets (df = degrees of freedom, SS = sum of squares, MS = mean squares, CoV = components of variance).

Dataset	Source	df	SS	MS	CoV (%)	Phi	p-value
p4	Between populations	3	59.672	19.891	-0.267		
	Within populations	72	1508.498	20.951	100.267		
	Total	75	1568.170	20.909	100.000	-0.003	0.694
p2	Between populations	1	15.249	15.249	-0.281		
	Within populations	34	546.063	16.060	100.281		
	Total	35	561.312	16.016	100.000	-0.003	0.625

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J. Phycol. **55,** 1226–1238 (2019)

DOI: 10.1111/jpy.12919

GENERALIST LIFE CYCLE AIDS PERSISTENCE OF *ALEXANDRIUM OSTENFELDII* (DINOPHYCEAE) IN SEASONAL COASTAL HABITATS OF THE BALTIC SEA[1]

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In seasonal environments, strong gradients of environmental parameters can shape life cycles of phytoplankton. Depending on the rate of environmental fluctuation, specialist or generalist strategies may be favored, potentially affecting life cycle transitions. The present study examined life cycle transitions of the toxin producing Baltic dinoflagellate *Alexandrium ostenfeldii* and their regulation by environmental factors (temperature and nutrients). This investigation aimed to determine whether genetic recombination of different strains is required for resting cyst formation and whether newly formed cysts are dormant. Field data (temperature and salinity) and sediment surface samples were collected from a site with recurrent blooms and germination and encystment experiments were conducted under controlled laboratory conditions. Results indicate a lack of seasonal germination pattern, set by an endogenous rhythm, as commonly found with other dinoflagellates from the Baltic Sea. Germination of quiescent cysts was triggered by temperatures exceeding 10°C and combined nutrient limitation of nitrogen and phosphorus or a drop in temperature from 16 to 10°C triggered encystment most efficiently. Genetic recombination was not mandatory for the formation of resting cysts, but supported higher numbers of resistant cysts and enhanced germination capacity after a resting period. Findings from this study confirm that *A. ostenfeldii* follows a generalist germination and cyst formation strategy, driven by strong seasonality, which may support its persistence and possibly expansion in marginal environments in the future, if higher temperatures facilitate a longer growth season.

Key index words: **Cyst; dinoflagellate; dormancy; encystment; germination; microalgae; quiescence; resting cyst; sexual reproduction**

***Abbreviations*: a, *Alexandrium ostenfeldii* strain AOB202; ab, combination of two *Alexandrium ostenfeldii* strains: AOB202 and AOB348; ac, combination of two *Alexandrium ostenfeldii* strains: AOB202 and AOB504; b, *Alexandrium ostenfeldii* strain AOB348; bc, combination of two *Alexandrium ostenfeldii* strains: AOB348 and AOB504; c, *Alexandrium ostenfeldii* strain AOB504; C, control; mix, combination of five *Alexandrium ostenfeldii* strains: AOB202, AOB348, AOB504, AOB413, and AOB329; N, nitrogen limitation; NP, nitrogen and phosphorus limitation; P, phosphorus limitation; T, drop of temperature from 16 to 10°C**

INTRODUCTION

Life in the Baltic Sea must cope with strong seasonal fluctuations of environmental factors, such as temperature, light, and nutrients. When environmental conditions become unsuitable for growth, organisms must respond accordingly to endure. As part of many phytoplankton species' life cycle, resting cysts serve as a crucial component for survival (Ellegaard and Ribeiro 2018). Particularly in environments with a strong seasonality, where vegetative growth may be restricted to a certain time of the year, the recurrence and timing of species in the water column can largely depend on the formation and germination of resting cysts (e.g., Kremp et al. 2009 and references therein). Since benthic sediments in coastal areas can be rich in resting cysts,

[1]Received 15 February 2019. Accepted 29 August 2019. First Published Online 13 September 2019. Published Online 6 October 2019, Wiley Online Library (wileyonlinelibrary.com).
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Editorial Responsibility: S. Lin (Associate Editor)

they have important implications for plankton dynamics (e.g., Lundholm et al. 2011). Large deposits of long-term resting cysts in the sediment—so-called "seed banks"—were, for example, proposed to have an anchoring effect on aquatic microalgae populations, leading to increased genetic diversity and population differentiation (Sundqvist et al. 2018). Seed banks and benthic–pelagic coupling play a key role in harmful algal bloom formation, as they can lead to both locally restricted and large-scale regional blooms (Anderson et al. 2012).

Diverse phylogenetic phytoplankton groups form various types of resting stages with different purposes in the life cycle. Some resting stages, such as the hypnozygotes of dinoflagellates may be linked to sexual reproduction, whereas other cysts are asexual and may be formed as a response to parasite infection, grazing pressure, or environmental cues, which signal the end of the growth period (Anderson 1998, Anderson et al. 2003, Toth et al. 2004, Ellegaard and Ribeiro 2018). Although the formation of phytoplankton resting cysts plays a crucial role for their ecology and evolution (Ellegaard and Ribeiro 2018), requirements and triggers of their formation and germination remain unknown for many taxa. This information is available for ecologically important and toxic dinoflagellate species, where detailed studies on life cycle transitions have significantly contributed to a better understanding of bloom dynamics (Anderson 1998, Bravo et al. 2010, Ní Rathaille and Raine 2011). These studies indicate that habitat-specific environmental conditions require specific germination and encystment patterns, thereby shaping bloom strategies (Anderson 1998). In strongly seasonal habitats, environmental variability is high and suitable growth conditions can be restricted to a short time period. Germination outside of this time period can be inhibited by dormancy, a well-documented trait that helps microorganisms cope with environmental variability (Lennon and Jones 2011). "Dormancy" is defined as suspension of growth by endogenous regulation, requiring a period of physiological ripening (maturation or dormancy period), before cysts can germinate (e.g., Anderson 1998). Once released from dormancy cysts may enter a state of "quiescence," which is defined as suspension of growth due to external conditions (e.g., environmental factors; Anderson 1998). Internal (e.g., endogenous clock, or other) and/or external (e.g., temperature) factors regulate dormancy transitions, and when cysts are quiescent, certain temperature ranges can stimulate germination (Anderson and Rengefors 2006, Fischer et al. 2018). Temperature acts as a reliable signal by changing in predictable ways during the seasonal cycle, thereby setting the window for growth, depending on growth requirements of the respective species (Ellegaard and Ribeiro 2018). While dormancy and temperature primarily drive germination of seasonal dinoflagellates, cyst formation often depends on nutrient conditions, particularly when linked to sexual reproduction (Figueroa et al. 2008). The blooms of cold water dinoflagellates commonly occurring in the northern Baltic Sea during spring are regulated by dormancy, temperature, and nutrient-dependent life cycle transitions (Kremp et al. 2009).

Since the early 2000s, the toxin producing dinoflagellate *Alexandrium ostenfeldii* (Paulsen) Balech and Tangen (1985) has started to form dense, recurrent blooms during summer in shallow coastal waters of the Baltic Sea (Kremp et al. 2009). Like most other *Alexandrium* species, *A. ostenfeldii* forms resting cysts (Mackenzie et al. 1996, Anderson et al. 2012), which anchor Baltic bloom populations in their respective locations (Hakanen et al. 2012, Tahvanainen et al. 2012). The life cycle of *A. peruvianum*, a heterotypic synonym of *A. ostenfeldii* (Kremp et al. 2014), was studied in detail on isolates from the Mediterranean Sea (Figueroa et al. 2008). The authors found the life cycle of *A. peruvianum* to be very complex, with various alternative options of sexual and asexual reproduction and formation of different types of cysts. This suggests that the species follows a generalist life cycle strategy, which may increase its capability to withstand variable adverse conditions and could contribute to the ongoing expansion of *A. ostenfeldii* (Van de Waal et al. 2015). Weather generalists or specialists are more successful depends on temporal and spatial environmental variability and the scale of variation. Generalists are favored by temporal variability and heterogeneous environments, whereas specialists may benefit from spatial variability and homogeneous environments (Reboud and Bell 1997, Kassen 2002, Collins 2011, Crowley et al. 2016). Strong seasonal fluctuations of temperature, light, and nutrients prevail in of the Baltic Sea, suggesting that generalist strategies are favored. Compared to the northern Baltic Sea, Mediterranean lagoons do not have strong seasonal environmental fluctuations. As such, different geographic populations could have evolved different life cycle strategies that reflect the conditions of their respective habitats, as shown for some species (Anderson 1998, Hallegraeff et al. 1998). Therefore, it is plausible that, similar to the other seasonal dinoflagellates from the northern Baltic Sea (Kremp 2000, Kremp and Anderson 2000, Kremp and Parrow 2006), *A. ostenfeldii* from the Finnish coast has a pronounced dormancy period and a narrow temperature window for germination, pointing at increased specialization. Formation of resting cysts, which are resistant to bacterial degradation during prolonged periods of cold and dark conditions, should be a prerequisite to survive the harsh winter conditions and resume vegetative growth in spring. To test this, an investigation into the regulation of life cycle transitions in a local *A. ostenfeldii* bloom population was carried out using material collected from the field and from experiments with cultured isolates. Aims

of the project included defining the dormancy interval, delineating triggers for life cycle transitions and assessing how relevant genetic recombination is for the formation of resistant resting cysts (i.e., cysts that remain intact after 1 year of storage). To accomplish this, germination and encystment experiments were performed under controlled laboratory conditions. Resting cysts were isolated from sediment samples, collected repeatedly from a shallow embayment of the Åland archipelago at the SW coast of Finland. Clonal cultures were established to test encystment triggers and the relevance of genetic recombination. In addition, environmental parameters were monitored at the bloom site to assess the implications for the natural population.

MATERIALS AND METHODS

Sediment sampling and sediment processing. To quantify cysts in the sediment and obtain a cyst slurry for all further experiments, sampling took place at the Föglö archipelago, Åland islands. Kremp et al. (2009) and Hakanen et al. (2012) described the location in detail, which corresponds to station 4 in the latter reference. The shallow sound (water depth <3 m) has a muddy bottom and is partly densely vegetated. Summer salinity is typically 6–7, the estuary is usually ice-covered from December to April and in summer the water temperature may rise to +24°C. Replicate sediment cores were taken with a gravity corer (Limnos, Turku, Finland). The uppermost flocculent sediment layer was transferred from the middle of the cores to 50 mL centrifugation tubes. The tubes were entirely filled with sediment and stored at 4°C in the dark until further use to prevent germination of resting cysts before starting the experiments. These storage conditions were chosen to imitate winter conditions and have been applied successfully earlier (Jerney et al. 2019). Prior to the experiments ~ 5 mL sub-samples of the sediment were diluted to 10 mL with sterile local sea water with a salinity of 6 and sonicated for 30 s on constant duty cycle with a frequency of 20 kHz (Brandelin Sonoplus sonicator HD 2200) to detach resting cysts from sediment particles. Samples were cooled with ice to avoid temperature increase during sonication and sieved afterwards to isolate the 30–76 µm fraction, containing *A. ostenfeldii* resting cysts. The material retained on the 30 µm screen was transferred into a 15 mL polypropylene centrifuge tube and diluted with filtered sterile local seawater with a salinity of 6 to obtain a cyst slurry for quantification and isolation of single cysts.

Characterization of dormancy and seasonal germination capacity. To characterize dormancy behavior of northern Baltic *Alexandrium ostenfeldii*, two approaches were used: (i) Germination capacity was studied with cysts isolated from freshly sampled sediment (as described above), collected throughout the annual cycle in approximately monthly intervals from May 2010 to April 2011. Germination experiments, were set up with six replicates with 7–10 cysts each. Single cysts were isolated from the cyst slurry with a glass micropipette and transferred to wells of a 12-well tissue culture plate (Greiner Bio-One Cellstar) filled with f/8 medium (Guillard and Ryther 1962, Guillard 1975) made from sterile local seawater with a salinity of 6. Cysts were then incubated at 16°C, 16:8 hours light:dark cycle (light intensity ~ 60 µmol photons $\cdot m^{-2} \cdot s^{-1}$). After 2 and 4 weeks, the number of empty and full cysts was recorded microscopically with an inverted microscope (Leica DMI3000 B) to calculate the germination percentage. Monthly germination data were compared to *A. ostenfeldii* cell concentrations in the water to estimate the contribution of newly formed cysts to the cyst pool in the sediment and support interpretation of germination data (Fig. 1). *A. ostenfeldii* cell concentrations in the water were determined by microscopy of water samples fixed with Lugol's solution, as outlined in Hakanen et al. (2012). (ii) A second set of germination experiments was conducted using newly formed cysts sampled right after the bloom peak in September 2015. Three replicate sediment core samples were pooled, mixed, and aliquots for monthly germination experiments were stored at 4°C in the dark. The first germination experiment was set up 1 day after sampling and repeated every 2–6 weeks until April 2016 to check if cysts stored under constant conditions have the same germination capacity as freshly sampled cysts. For each experiment, one aliquot was taken from storage, processed as described above and examined for cysts. For each experiment, 50 single intact cysts were isolated into individual wells of a 96-well plate, containing 300 µL f/8-si medium and incubated at 16°C, 12:12 hours light:dark cycle (light intensity ~100 µmol photons $\cdot m^{-2} \cdot s^{-1}$). The well plates were examined for germinated cysts 2 and 4 weeks after start of each experiment and the germination percentage was calculated as for the first approach (Figure S1 in the Supporting Information). Most viable cysts usually germinate within 2 weeks (Jerney et al. 2019), but to ascertain that all capable cysts had germinated, the well plates were re-inspected after 4 weeks.

Determination of temperature requirements for germination. To determine the temperature range permitting germination of *Alexandrium ostenfeldii* cysts, a temperature gradient experiment with 10 different temperatures (4, 8, 10, 12, 14, 16, 18, 20, 22, and 24°C) was carried out in February 2018 with sediment samples collected in May 2017. Sediment was processed as described above and cysts were concentrated with the help of a density gradient using sodium polytungstate (Bolch 1997). For each temperature treatment, 30 cysts were isolated to three replicate wells (10 cysts per well) of a 96-well tissue culture plate (Greiner Bio-one microplates with µClear® bottom and white walls), containing 300 µL of f/8-Si. Isolation was done at 12°C room temperature to keep the cysts as cool as possible during the isolation process. Additionally, well plates were cooled on a plate heat exchanger set to 4°C, cooled by a temperature control unit (Lauda, Germany), and covered with aluminum foil during the isolation process to minimize potential light and temperature stimulation of the cysts before the start of the experiment. The cyst slurry was placed on ice and covered with aluminum foil during the isolation process for the same reason. To start the experiment, each well plate was placed on a separate plate heat exchanger connected to a temperature control unit, which was adjusted to one of the ten specific temperatures. To reduce evaporation, well plates were covered with a transparent plastic membrane, which was exchanged every second day to allow sufficient gas exchange. A custom-made incubator with 96 white light-emitting diodes fitted to the geometry of a 96-well tissue culture plate was placed on top of each culture plate for illumination and adjusted to 100 µmol photons $\cdot m^{-2} \cdot s^{-1}$ and a 14:10 hours light:dark cycle. The experiment lasted for 3 weeks and empty (germinated) and full (not germinated) cysts were counted with an inverted microscope (Leica DMI3000 B) once a week to calculate the percentage of germination (Fig. 2a). To relate temperature requirements for germination to seasonal temperature dynamics at the bloom site, temperature was recorded at the sediment surface (Fig. 2b) with a HOBO Pendant Temperature/Light Data Logger (Onset, Bourne, MA, USA), deployed at a water depth of 1.3 m. The data logger was attached to a steel plate, which was partly submerged in the soft sediment to prevent displacement of the logger. The temperature was recorded

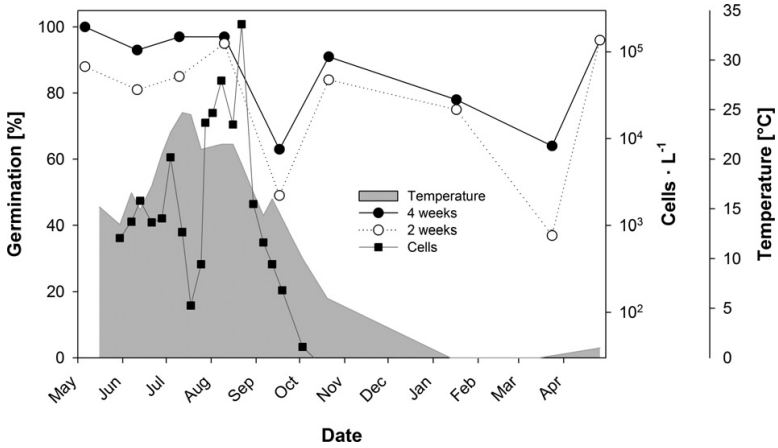

FIG. 1. Cumulative germination of resting stages, isolated from sediment sampled repeatedly throughout the year (from May 2010 to April 2011), 2 and 4 weeks after isolation (means, $n = 6$); cell numbers of vegetative cells in the water column (logarithmic scale) and temperature at the water surface.

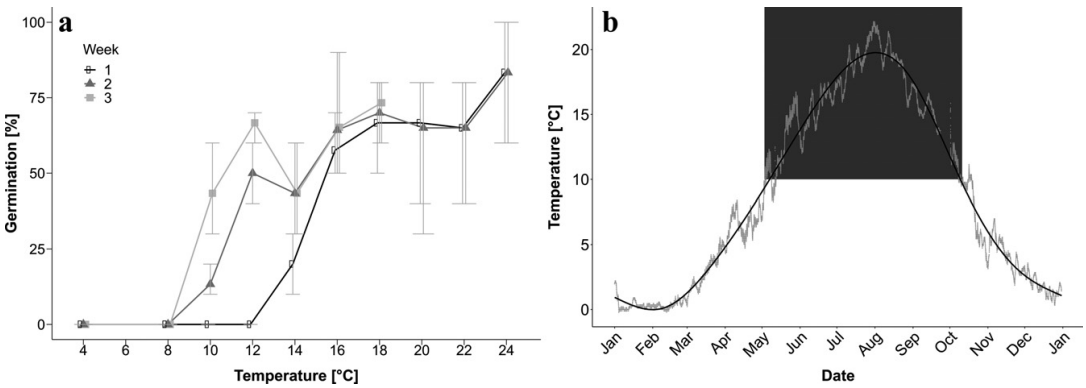

FIG. 2. (a) Germination capacity of resting cysts collected from the field at different temperatures, after 1, 2, and 3 weeks of incubation (means ± SD, $n = 3$). (b) Sediment surface temperature at a bloom hotspot during 2017. The black line represents a generalized additive model and the gray background area indicates the temperature regulated germination interval of *Alexandrium ostenfeldii* in the Baltic Sea (restricted to temperatures above 10°C).

every 10 min from January 2017 to January 2018. Since the sampling site is very shallow and the entire water column mixed, it can be assumed that sediment surface and water surface temperatures are very similar. Therefore, temperature was measured at the water surface from 2010 to 2011.

Encystment experiments. To define triggers and the relevance of genetic recombination for cyst formation, experiments were carried out testing the effects of different nutrient and temperature conditions on cultured clonal strains and crosses of clonal strains. Cultured clonal isolates of *A. ostenfeldii* used in these experiments were established in June to August 2015 (Jerney et al. 2019). Cultures were grown in 50 mL tissue culture flasks, filled with 40 mL f/2-Si enriched local sea water at salinity of 6, 16°C–20°C, 100 µmol photons $\cdot m^{-2} \cdot s^{-1}$ and a 14:10 hours light:dark cycle until the beginning of the experiment. The five experimental cultures were established from different sampling dates to reduce the risk of choosing sibling strains.

Single strains AOB202 (a), AOB348 (b), AOB504 (c), a combination of two strains (ab, ac, and bc), and a mix of five strains (mix = a, b, c, AOB413, and AOB329) were used for all encystment treatments to account for a potential requirement of genetic recombination. Inoculum cultures for the different experiments were grown in 500 mL of f/2-Si medium at 16°C to reach concentrations of ~10,000 cells $\cdot$ mL^{-1} (exponential phase). Triplicate experimental units of 200 mL were inoculated with equal amounts of single strains and mixes at a starting concentration of ~1,000 cells $\cdot$ mL^{-1}. Encystment was tested at 16°C with f/2-Si medium, serving as control (C) and medium with reduced nitrate (N), phosphate (P), or both nutrients (NP) to 10% of the f/2-Si level. In addition, one temperature treatment was tested, inoculating experimental cultures, pre-grown in f/2-Si medium at 16°C,

into 10°C f/2-Si medium (T). To record cell and cyst dynamics in the experimental cultures, culture flasks were gently shaken for a homogeneous cell distribution and 1.1 mL of sample was taken into an Eppendorf vial, preserved with a drop of neutral Lugol's solution. Samples were taken twice a week and cells and cysts were counted using a 1 mL Sedgewick Rafter counting chamber and an inverted microscope (Leica DMI3000 B). After 6 weeks of regular sampling, the experiments were terminated and cysts that had accumulated at the bottom of the flasks were harvested. The majority of the medium was aspirated and culture flasks and the remaining cyst slurry were shaken roughly to detach sedimented cysts from the flask bottom. Replicates were pooled into a 50 mL glass flask and sonicated for 1 min to destroy remaining cells. Afterwards cysts were concentrated with a 30 μm sieve, rinsed with filtered seawater, and transferred to 50 mL centrifugation tubes, which were filled up to 45 mL with filtered seawater. Total cysts formed in experimental flasks were enumerated from these samples and cyst yields were calculated by dividing the number of harvested cysts at the end of the experiment by the maximum cell number recorded during the growth period (Fig. 3). Another set of subsamples was transferred to 2 mL cryovials, covered with a 10 μm mesh net, closed with a drilled cap and submerged bottom-up in anoxic sediment, which was stored at 4°C in the dark as described by Anderson et al. (2003) for later use.

To see whether resting cysts produced under different conditions differ morphologically from each other, micrographs were taken with a digital camera (Leica DFC490) attached to an inverted microscope (Leica DMI3000 B) after harvesting cysts at the end of the encystment experiment. Micrographs of 30 cysts from single strains and strain combinations of each treatment were inspected and compared to pictures taken after 1 year of storage (Fig. 4). Pale, disintegrated cysts with an irregular outline were considered degraded after storage, indicating that they were not resistant to bacterial degradation.

Germination of experimentally produced cysts. A first germination experiment was set up two days after termination of the encystment experiment, to test if internal maturation is necessary before resting cysts can germinate. For this purpose, 2 mL cryovials were taken from the anoxic storage, resuspended in sterile filtered sea water and 1 mL of the resulting cyst slurries were transferred to replicate 24-well plates, containing f/8-Si medium. Cysts were incubated for 14 d at 16°C at a light intensity of ~ 70 μmol photons m^{-2} s^{-1}, 14:10 hours light:dark cycle. Germination experiments were repeated weekly or biweekly for 2 months. Cysts produced by all single strains, as well as the five strain mixes from the reduced NP and the T treatment were included in this experiment (Figure S2 in the Supporting Information). After 1 year of storage at 4°C in the dark, cyst concentrations in storage containers were determined again and germination capacity was tested as described above, though incubations were carried out in smaller volumes in eight replicate wells of a 96-well plate, containing 200 μL f/2-Si medium. Wells were scored positive for germination when swimming cells were observed after 1 and 2 weeks (Fig. 5).

Statistics. Data exploration, basic calculations, and plotting of graphs were done with R (R Core Team 2018), RStudio (RStudio Team 2015), and Sigma Plot V14.0 (Systat Software, San Jose, CA, USA). Smoothed conditional means were added to the sediment surface temperature data in Rstudio with the help of the ggplot2 package (Wickham 2009) using a generalized additive model from the nlme package (Pinheiro et al. 2018) as a smoothing method.

RESULTS

Seasonal germination and dormancy dynamics. *A. ostenfeldii* resting cysts germinated throughout the year from freshly sampled sediment between May 2010 and 2011 (Fig. 1). The final germination success was

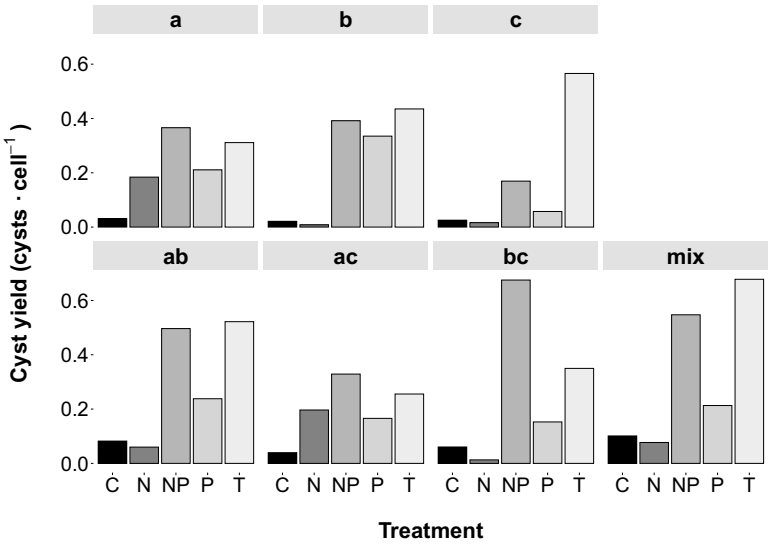

FIG. 3. Cyst yields (number of harvested cysts at the end of the experiment divided by the maximum cell number during the growth period) of *Alexandrium ostenfeldii* clones and mixes of clones at different encystment treatments: Control (C), nitrogen limitation (N), phosphorus limitation (P), combined nitrogen and phosphorus limitation (NP), and reduced temperature (T). Single strains (a, b, and c) are shown in the upper row, combinations of two (ab, ac, and bc) and five strains (mix) in the lower row.

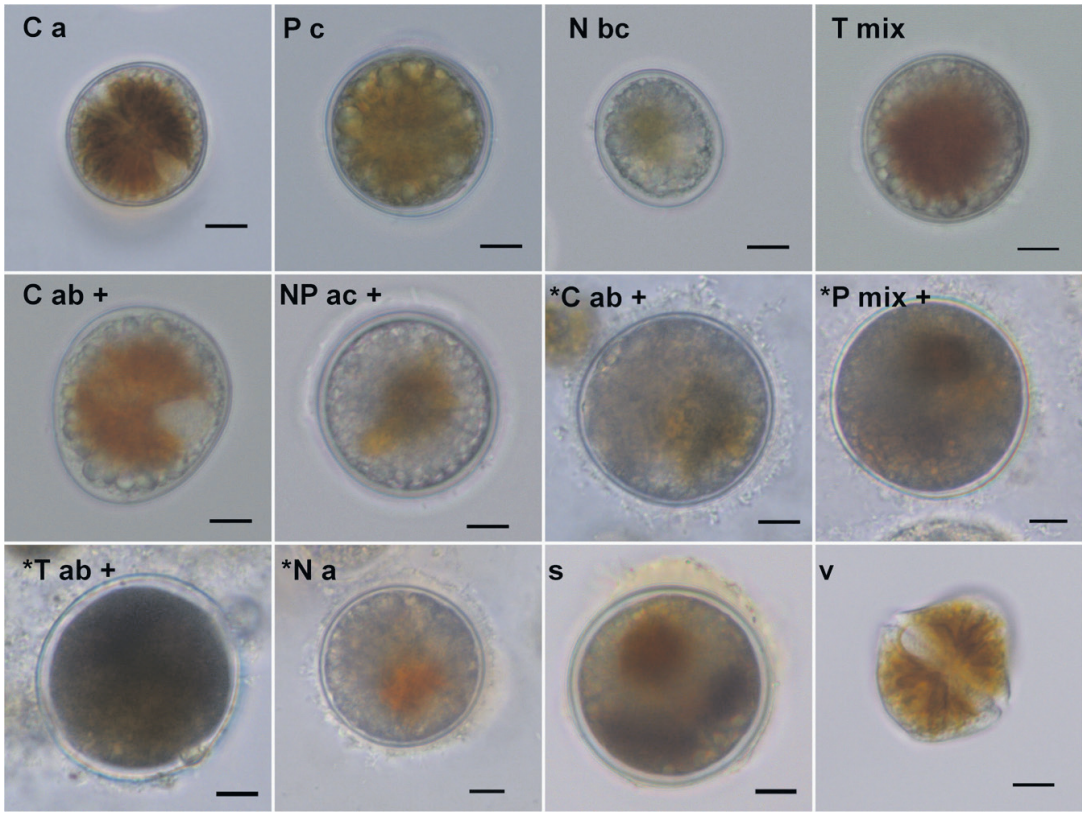

FIG. 4. Morphological variability of *Alexandrium ostenfeldii* resting stages before and after storage for 1 year at 4°C in the dark. Capital letters indicate the treatments: Control (C), nitrogen limitation (N), phosphorus limitation (P), combined nitrogen and phosphorus limitation (NP), and reduced temperature (T). Small letters indicate single strains (a and c) or combinations of two (ab, ac, and bc) and five strains (mix). Micrographs taken after 1 year of storage at 4°C are labeled with a star (*) and successful germination with a plus symbol (+). Typical cyst found in sediment samples from Föglö archipelago (s) and a vegetative cell, grown at control conditions (v). Scale bars 10 μm. [Color figure can be viewed at wileyonlinelibrary.com]

above 90% from May to August and in November, after 4 weeks of incubation. Shortly after the bloom peak in the end of August, germination declined to 49% but increased to 63% in September. Slightly reduced germination was also observed in February and April, but the proportion of germinated cysts remained above ca. 60%, after 4 weeks of incubation throughout the year. Dormancy dynamics was not coupled with seasonal dynamics of cells in the water column. Cells appeared at the end of May, when water temperature was above 13°C and cell numbers remained low (<5,000 cells L^{-1}) until June. A maximum temperature of 24.7°C was measured in July and temperature remained around 20°C during a bloom, developing in August when cell concentrations rose to approximately 50,000 cells $\cdot L^{-1}$. In August, peak concentrations of >200,000 cells$\cdot L^{-1}$ were measured, before the cell concentration dropped suddenly below 2,000 cells$\cdot L^{-1}$ at the end of August during a decreasing temperature trend (around 16°C). Cells disappeared from the water completely in October, when the temperature went below 10°C.

Germination of *A. ostenfeldii* resting cysts was not inhibited endogenously during the winter months, as shown by successive biweekly to 6-weekly incubation experiments with cysts collected shortly after the bloom peak in September 2015 (Figure S1). Germination was possible for more than 70% of the cysts, within 4 weeks of incubation, throughout the winter months until March 2016, although reduced to ca. 50% shortly after the bloom peak in September 2015.

Temperature window for germination. Germination of *Alexandrium ostenfeldii* was completely inhibited by temperatures below 10°C (Fig. 2a). Delayed germination occurred at 10°C conditions with a mean cumulative germination below 50%. Between 10°C

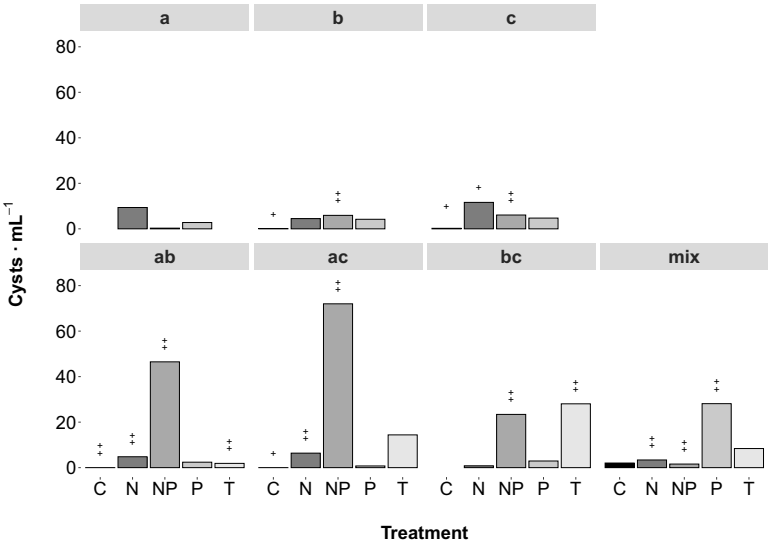

FIG. 5. Concentration of resting cysts per ml of initial culture medium, produced in the laboratory, by different strains and strain combinations, after 1 year of storage (4°C, dark). The encystment conditions were as follows: Control (C), nitrogen limitation (N), phosphorus limitation (P), combined nitrogen and phosphorus limitation (NP), and reduced temperature (T). Single strains (a, b, and c) are shown in the upper row and combinations of two (ab, ac, and bc) and five strains (mix) in the lower row. Crosses above the bars indicate if vegetative cells were present 1 week (+) and 2 weeks (++) after re-exposure to suitable growth conditions.

and 14°C germination percentage increased, but was still lower compared to 16°C and 24°C, where most viable cysts germinated within 1 week. The highest germination success of more than 75% on average was recorded at 24°C. Longer monitoring periods might have eventually revealed higher germination rates at lower temperatures but the experiments were terminated after 3 weeks because few additional cysts germinating even later were considered ecologically less relevant for bloom formation in nature.

As depicted in Figure 2b, the water temperature at the sediment surface of our sampling location showed a strong seasonal variation between 0°C in February and 22°C at the end of July. The temperature threshold of 10°C, for germination of *A. ostenfeldii,* was exceeded for the first time in the beginning of May, and temperature fell below this value in October, which allows cysts to contribute to the bloom inoculum over a period of 6 months, from May to October.

Regulation of encystment. Encystment experiments with cultured *A. ostenfeldii* isolates showed that cyst formation is regulated by multiple factors. NP and T resulted in highest cyst yields in clonal cultures and strain combinations. The highest yield of 0.67 cysts per vegetative cell was reached in the mix, treatment T and in the bc strain combination, treatment NP (Fig. 3). T induced the highest cyst yield for single strains b and c, strain combination ab and the mix. In contrast, NP triggered highest cyst yields for strain a and the strain combinations of ac and bc. Treatment P led to low or intermediate cyst yields for all strains and strain combinations, ranging between 0.15 (bc) and 0.33 (b) cysts per vegetative cell. The least effective trigger for cyst formation was N (strains b, c, ab, bc, and mix). In two strain pairs (ab and bc) and the mix, cyst yields at NP and T were higher than in single strains.

Morphology, survival, and germination capacity of experimentally produced cysts. Cyst morphologies varied substantially between the treatments N, P, NP, and T (Fig. 4). No pronounced morphological differences related to single strains or strain combinations were found by visual inspection and cell size measurements (data not shown). Variable cyst shapes (spherical to slightly elongated), cyst wall thicknesses, and cyst sizes were observed. Reduced pigmentation and the occurrence of a red accumulation body were associated with cyst formation triggers: Cysts formed under C and T were strongly pigmented and had on average few thick-walled cysts (14% and 12.5%). More thick-walled cysts were formed under N, NP, and P (23, 27 and 23.5%), but in total the majority of cysts were thin-walled (80%). Cysts formed at N and P had thicker walls compared to the other treatments, despite centered, weak pigmentation. To see whether the cyst morphology changes over time, micrographs of cysts before and after (Fig. 4, star symbol) storage were compared. Cyst morphology was different from

newly formed cysts after 1 year of storage because mainly spherical and no elongated cysts were found. Although there were many degrading, pale cysts, most of the intact cysts had a strong pigmentation.

To compare the germination capacity of freshly produced and stored cysts, germination experiments were set up 2 days and 1 year after harvesting cysts of the encystment experiment. Around 40%–70% of the freshly produced cysts germinated within 7 d, independent of the encystment trigger and strain combination (Figure S1). Cysts that resembled wild cysts (Fig. 4s) had the best germination capacity after storage, independent of the treatment. Compared to single strains strain combinations produced higher numbers of resistant cysts, with a higher germination capacity after storage (Fig. 5). NP and combinations of two strains resulted in the highest number of cysts after storage: 1.14% (ac) and 0.87% (ab) of cells formed cysts, equaling 72 cysts · mL^{-1} (ac) and 47 cysts · mL^{-1} (ab). Fewer cysts (20–30 cysts · mL^{-1}) were formed by P (mix), NP, and T (bc), equaling 0.55, 0.43, and 3.1% of cysts per cell. A high percentage (3.1%) of cysts found in the bc strain mix of treatment T is related to low cell numbers due to slow growth at the low temperature. The number of viable cysts was much lower for single strains after 1 year of storage (below 12 cysts · mL^{-1}, or 0.2 % cysts per cell) and only cysts produced by single strains b and c germinated and grew thereafter (indicated by two crosses above bars in Fig. 5).

DISCUSSION

This study demonstrates that the majority of the Baltic *A. ostenfeldii* resting stages are quiescent throughout the year. Newly formed resting cysts have a very short maturation period before possible germination and no pronounced dormancy interval, as it is typical in seasonal bloom forming Baltic dinoflagellates (Kremp 2000, Kremp and Anderson 2000, Kremp and Parrow 2006). Inhibition of quiescent cysts occurred as a result of temperatures below 10°C in our experiments. Suitable germination temperatures, corresponding to the occurrence of the species in the water column, lasted from May to October. Temperature reduction to 10°C and NP limitation of nitrogen and phosphorus were the strongest encystment triggers. More resistant cysts were formed when multiple strains were combined, especially when cyst formation was triggered by combined phosphorus and nitrogen limitation. In addition, a variety of treatment-related cyst morphologies were observed.

Regulation of dormancy and germination. Tightly linked dormancy and temperature control of cyst germination often determine the growth phase of dinoflagellates in seasonal habitats (Ellegaard et al. 1998, Kim and Han 2000, Anderson and Rengefors 2006). The strong seasonality of the Baltic Sea prevents growth of warm-water-adapted *A. ostenfeldii* during the winter months and blooms usually occur between July and August (Hakanen et al. 2012). Accordingly, the expectations for this study included finding a winter dormancy period and a summer germination temperature interval. However, results of this study reveal a different germination strategy for the species: a lack of dormancy during the winter months, coupled with moderate temperature control, preventing germination at water temperatures below 10°C. This temperature control can be considered moderate, compared to other seasonal dinoflagellates, which have more narrow temperature ranges controlling germination (Rengefors and Anderson 1998, Kremp and Anderson 2000). Similarly, a wide range of temperatures (12°C–28°C) allowed high germination rates of *A. catenella (= A. tamarense)* from Thau lagoon, France (Genovesi et al. 2009). The authors suggested that the observed seasonal germination pattern was independent of temperature fluctuations. The study presented here shows that the majority of *A. ostenfeldii* cysts in the sediment are quiescent and ready to germinate throughout the year, as soon as favorable conditions are provided. Conditions are considered favorable if they support vegetative growth and reproduction (e.g., temperatures above 10°C, sufficient light and nutrients). Reduced germination capacity of the cyst population was detected shortly after the bloom peaked in September 2011 (Fig. 1) and 2015 (Figure S1), which could reflect the recent deposition of newly formed, immature cysts decreasing the fraction of total cysts capable of germination in the investigated pool. The fact that the same pattern was found in both years supports this interpretation. The slightly reduced germination capacity in April 2011 is inconsistent with the rest of the data due to high germination success earlier in winter and in May 2012. This inconsistency possibly reflects the natural variability of germination in the population, which can be high, spatially and temporally, when not all genotypes follow the same strategy (Anderson 1998). Alternatively, several germination strategies might exist in the Baltic *A. ostenfeldii* population and partly explain the observed seasonal variation. Continuous germination after a short maturation period and germination controlled by an circannual internal rhythm (preventing germination of dormant cysts) could both exist as alternative strategies in the same population, as suggested for other seasonal *Alexandrium* species (Perez et al. 2002). Coexistence of several germination (emergence) strategies was proposed to result from different levels of temporal environmental variability (De Stasio and Hairston 1992, Martínez-García and Tarnita 2017) and can ensure survival in case the majority of a population germinates, but is lost afterwards due to unfavorable environmental conditions (Anderson 1998).

As reported for *A. catenella (= A. tamarense)* from Thau lagoon, France, (Genovesi et al. 2009), *A. ostenfeldii* in this study required a very short maturation period after cysts were formed, before they could germinate again. The interpretation of field data is supported by the results of laboratory experiments which showed that cysts derived from encystment experiment (Fig. 3) were able to germinate at a high rate within 7–14 d once suitable conditions were restored (Figure S1). Interestingly, very short maturation periods of 10–20 d were reported for a Chinese *A. ostenfeldii* strain from Bohai Sea (Gu 2011) which is genetically closely related to the Baltic Sea population (Kremp et al. 2014), but experiences different habitat conditions. In addition, a short maturation period of less than 10 d for Mediterranean *A. ostenfeldii* sensu Kremp et al. (2014) strains was reported (Figueroa et al. 2008), suggesting that the duration of maturation in *A. ostenfeldii* may, to some extent, be genetically predetermined.

In contrast to Baltic *A. ostenfeldii*, prolonged dormancy periods, lasting several months, can inhibit germination in other seasonal *Alexandrium* spp. (e.g., Anderson 1980, Kim et al. 2002, Mardones et al. 2016, Fischer et al. 2018). Also resting cysts of Mediterranean populations of *A. ostenfeldii* sensu Kremp et al. (2014) have a dormancy period of numerous months (Figueroa et al. 2008) although habitat conditions at the Catalan coast are stable compared to the Baltic Sea. Correspondingly, variable dormancy periods of one and the same species, but different geographic isolates, were found for *A. catenella* (Hallegraeff et al. 1998). According to Hallegraeff et al. (1998), different ecological roles of cysts, such as overwintering strategy versus rapid cycling between benthos and plankton, might be responsible for varying cyst dormancy requirements.

Temperature is an important factor in controlling dinoflagellate cyst germination by maintaining quiescence for extended periods, determining the duration of dormancy after cyst formation, synchronizing germination or initiating the excystment process (Anderson 1998 and references therein). The data presented here show that a defined temperature window for germination of Baltic *Alexandrium ostenfeldii* exists, ranging from 10°C to 24°C (Fig. 2a). At lower temperatures (10°C–14°C), the average germination success is reduced, which indicates that temperature functions as environmental filter, preventing germination of some part of the cyst population. A broad temperature range permitting germination is in line with earlier findings, reporting that temperature does not affect germination success between 16°C and 20°C (Jerney et al. 2019). Lower temperatures reduced germination of *A. tamarense* from Irish coastal waters, whereas excystment of *A. minutum* from the same habitat showed no temperature effect with almost equal success between 5°C and 25°C (Ní Rathaille and Raine 2011).

The temperature requirements of Baltic *A. ostenfeldii* restrict germination to the period between May and October (Fig. 2b), which is when cells of *A. ostenfeldii* can indeed be found in the water column (Fig. 1; Hakanen et al. 2012). A comparable temperature range was determined for the Chinese isolate from Bohai Sea (Gu 2011) and also the quicker germination at higher temperatures described by these authors is similar to our results (Fig 2a). These similarities further emphasize the close relationship of Baltic and Bohai Sea populations indicated by genetic data (Sildever et al. 2019).

Triggers of cyst formation. T and NP are the most effective triggers of northern Baltic *A. ostenfeldii* encystment (Fig. 3). Significant cooling of surface water—that is rapid decrease of temperature by several degrees—should be perceived by the organism as a signal of impending unfavorable growth conditions and trigger a protective response. In the northern Baltic, increasing temperature induced formation of dormant resting cysts of several cold water dinoflagellates (Kremp et al. 2009) which usually bloom in spring (Kremp and Heiskanen 1999). Thus, it is anticipated that a respective reverse temperature change triggers encystment of a species, which forms blooms in summer (Hakanen et al. 2012). Limiting concentrations of inorganic N and P do not seem to be directly related to bloom termination involving cyst formation in the field (Hakanen et al. 2012), but might represent an alternative strategy to ensure the deposition of resistant resting cysts in case temperature would not drop early enough before other conditions become unfavorable. Single nutrient limitation was less effective in triggering cyst formation than the other treatments, and P resulted in higher cyst yields than N. Despite the many similarities of the Chinese isolate with Baltic populations, the effect of P is inconsistent with the findings of Gu (2011), showing a higher cyst induction of the Chinese strain by N compared to P.

All single strains and strain combinations formed resting cysts (Fig. 3), which indicates that resting cysts can be formed with and without genetic recombination (i.e., by heterothallic and homothallic sexual reproduction). In contrast, planozygote and cyst formation of *Alexandrium minutum* was, for example, reported to be affected by low P/N ratios, low concentration of both nutrients and by salinity and temperature (Figueroa et al. 2011). Cyst yields of this study were higher in strain mixes than in single strains, possibly indicating heterothallic sexual reproduction, but the effect was not dramatic. Conclusions about the rate of genetic recombination of Baltic *A. ostenfeldii* strains remain speculative at this point because the development of single cells was not followed to trace and quantify zygote formation. Variable mating systems, with homothallic and heterothallic sexuality occurring in one and the

same species, have been described for other dinoflagellates (Figueroa et al. 2010) and also the formation of dormant resting cysts without sexual reproduction might be more common than previously assumed (Kremp and Parrow 2006). Zygote formation by clonal strains was observed in *A. ostenfeldii* cultures previously (Jensen and Moestrup 1997, Figueroa et al. 2008, Gu 2011) and underlines the assumption that many cases of sexuality reported in the literature may be the result of self-fertilization (Jensen and Moestrup 1997).

Observations from this study showed that Baltic *A. ostenfeldii* form a large diversity of cyst types, which is in line with earlier findings (Figueroa et al. 2008). The morphological characteristics of cysts, such as shape, thickness of the cyst wall, pigmentation, and size, seemed to be associated with encystment triggers rather than sexuality or the strain identity. In addition, the morphology of freshly produced cysts did not lead to conclusions about their resistance, since cysts of variable morphology showed different storage and germination capacities. Though thick-walled cysts are typically regarded as resistant resting cysts, relatively thin-walled cysts germinated in experiments after 1 year of storage (Fig. 4). Thin-walled cysts of other dinoflagellates have been demonstrated to function as dormant survival cyst, preserving the cell from degradation (Kremp 2000).

Results of the 1-year storage experiment, showing higher resistance and germination capacity of combined strain incubations (Fig. 5), suggest that genetic recombination could increase survival and resistance of cysts. Single strains tend to produce more temporary cysts, as documented in other studies (Jensen and Moestrup 1997, Figueroa et al. 2008). This study therefore supports the view that sexual reproduction and cyst formation can be independent processes (Bravo et al. 2014). The decoupling of cyst formation and sexual reproduction could facilitate increased asexual reproduction, which was proposed to become selected in marginal environments (Eckert 2002). When analyzing the genetic structure of several Baltic *Alexandrium ostenfeldii* populations, a previous study reported significant multilocus disequilibrium, indicating reduced sexual reproduction (Tahvanainen et al. 2012).

The role of temporary encystment. A high germination capacity throughout the year is unexpected for Baltic *A. ostenfeldii* as it seems to be a risky strategy in a temporally variable environment with frequent short-term fluctuations, in addition to seasonal variation. A large part of the population could be lost if favorable temperature conditions do not persist long enough for survival and reproduction. As shown in Figure 2b, temperature fluctuations of several degrees happen frequently throughout the year and it is presumable that temperature can rise above 10°C already in spring, but decrease thereafter again below this value. Repeated losses of a large fraction of cells germinating at the wrong time could exploit the seedbank and have severe long-term consequences, eventually resulting in extinction of a population. Prevention of germination under favorable conditions could reduce this risk, in the event that environmental conditions turn unfavorable again (Donohue et al. 2010). Large temporal variance in survival and reproductive stress favors dormancy, which is commonly thought of as an adaptation to variable environments (Venable and Brown 1988). On the other hand, dormancy would be less important if only a small fraction of cysts, located in the uppermost oxygenated sediment layer of the seed bank, can germinate (Anderson et al. 2012) or if environmental conditions do not fluctuate frequently. Cyst flux data are not available from the Föglö area, but the uppermost centimeter of the sediment is usually well oxygenated and flocculent, indicating that a large fraction of cysts could germinate under suitable conditions. Alternatively, the germination of other seasonal *Alexandrium* populations, shown to be independent of temperature and an endogenous clock, was suggested to be governed by secondary dormancy (Ní Rathaille and Raine 2011). Secondary dormancy is mediated environmentally and can prevent germination of mature cysts during periods that are favorable for germination but are not favorable for growth (Ní Rathaille and Raine 2011), whereas an endogenous annual clock regulates germination internally, irrespective of environmental conditions (Anderson and Keafer 1987). However, if life history transitions (encystment and germination) are initiated rapidly and are coupled with a quick turnover between the benthic and the pelagic phase via temporary encystment, survival, and reproductive success might not be compromised in variable environments; thus, dormancy becomes less relevant. Temporary asexual cysts can be frequently observed in ageing cultures of Baltic *A. ostenfeldii* and were also reported in Mediterranean and Danish strains (Jensen and Moestrup 1997, Figueroa et al. 2008).

Generalist life cycle strategy and global warming. With respect to their food or habitat preferences, species are considered generalists if they have broad preferences and specialists are defined as organisms with restricted use of habitats or resources (Ricklefs 1999). To expand this terminology to the life cycle, a generalist can be defined as species with broad range of possible life cycle transitions and triggers inducing them, whereas a specialist is restricted to a specific strategy and a specific trigger. According to this definition, for example, *Pseudo-nitzschia* spp. can be regarded as specialists because members of this genus follow one very specific reproduction strategy without known alternative life cycle options (von Dassow and Montresor 2011). In contrast, the versatility of encystment triggers, cyst types as well as germination and survival capacities of produced cysts

support the view that Baltic *A. ostenfeldii* follows a generalist life cycle strategy. Although cyst formation is required as an overwintering strategy, pointing at increased specialization, alternative life cycle routes exist and transitions are regulated by a broad range of triggers. In addition to flexible germination behavior, Baltic *A. ostenfeldii* populations are characterized by high plasticity and a great adaptation potential, based on large genotypic and phenotypic diversity, which most likely aids its adaptation and persistence under predicted future climate conditions (Kremp et al. 2016, Jerney et al. 2019). Due to global warming, an overall reduction of sea surface salinity and increase of mean sea surface temperature are expected in the future (e.g., Meier et al. 2012), which could aid expansion of this species (Kremp et al. 2012). Alternatively, expansion of *A. ostenfeldii* could be limited by increasing hypoxia in coastal zones of the Baltic Sea (Conley et al. 2011), since germination of dinoflagellates requires oxygen (e.g., Rengefors and Anderson 1998, Kremp and Anderson 2000). Recent studies showed that the beneficial effect of higher temperature on growth might be compromised by decreased salinity (Jerney et al. 2019), which makes predictions about future bloom development challenging. Furthermore, bloom formation depends on many other variables, like competition with other co-occurring phytoplankton, predation pressure or the availability of nutrients and convincing evidence that climate change will enhance the growth of harmful algal bloom species, like *Alexandrium*, over the far larger pool of competing other phytoplankton species is lacking (Wells et al. 2015).

CONCLUSIONS

This study concludes that the majority of the *A. ostenfeldii* resting cysts are quiescent throughout the year and germination is not inhibited by endogenous regulation during winter. Results indicate that newly formed cysts undergo a short maturation period of around 1 month, after which they are ready to germinate as soon as conditions become favorable. Temperature regulated germination is possible above 10°C and accelerated above 16°C. In shallow inlets of the Föglö archipelago inhabited by *A. ostenfeldii*, temperature of the sediment surface shows a strong seasonal variation which restricts germination and the occurrence of vegetative cells in the water column up to 6 months (from May to October). In the future, this germination period will likely expand due to an increase in the mean sea surface temperature by global warming (Meier et al. 2012). At the end of the growth season, cyst formation is likely triggered by a combined effect of nutrient limitation and a temperature drop below 10°C. This study shows that genetic recombination is not strictly required for the formation of resting cysts, but it strongly enhances formation of resistant cysts and resurrection capacity after a resting period. Life cycle transitions of *A. ostenfeldii* seem to be very flexible, indicating that this species follows a generalist life cycle strategy. A generalist strategy might support this species persistence and possibly expansion in geographically and ecologically marginal environments in the future if higher temperatures facilitate a longer growth season. Populations in marginal ecosystems are often isolated and under extreme selection pressures, like exposure to harsh physical conditions, environmental gradients, and anthropogenic impacts, resulting in anomalous genetics (Johannesson and André 2006). To fully understand the bloom dynamics of this species, quantification of cyst fluxes (including sedimentation and re-suspension rates) should be assessed in the future. Understanding the life cycle of resting cyst forming phytoplankton species and mechanisms affecting life cycle transitions is crucial because it will affect the interpretation of cyst records from the past. As demonstrated recently, past cyst records do not always match phytoplankton monitoring data (Kremp et al. 2018), which could be related to different modes of cyst formation, affecting their resistance to bacterial degradation. Another important aspect of alternative life cycle strategies is the sedimentation of different types of cysts and their fate—either burial of resistant resting cysts or degradation of cells and temporary cysts—which will affect biogeochemistry of the sediment and nutrient fluxes (Spilling and Lindström 2008). Once the life cycle of species is fully understood, modeling studies can be carried out to follow bloom trends and predict future bloom development (Lee et al. 2018).

ACKNOWLEDGMENTS

We thank Kaisu Jussila for her vigorous support of the experimental work and microscopy and Johan Franzén for assistance with sampling and field work. This work was funded in part by grants from the Academy of Finland (grant 310449 to J. J., S. S. and A. K.), Walter and Andrée de Nottbeck Foundation (J. J.), and Maj and Tor Nessling Foundation (P. H.).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: JJ, AK, and SS. Carried out sampling: JJ, PH. Performed the experiments: JJ, PH, SAA, AK, SS. Analyzed the data: JJ. JJ led the writing of the manuscript with input from all authors.

DATA SHARING AND DATA ACCESSIBILITY

Data will be accessible at Dryad.

PROTECTION OF HUMAN SUBJECTS AND ANIMALS IN RESEARCH

Not relevant for this study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Germination of *Alexandrium ostenfeldii* cysts, isolated from sediment sampled in September 2015 during a bloom peak (~11×10^3 cells · L^{-1}) and stored afterwards in aliquots, at 4°C in the dark. Every 2–6 weeks 50 cysts were isolated and inoculated at suitable growth conditions to check for germination two and four weeks later.

Figure S2. Germination percentage of resting stages, produced under controlled conditions in the laboratory, plotted against the number of storage days. Germination experiments were started two days after harvesting of cysts and repeated every 7–14 d (means, $n = 3$). Cysts were stored at 4°C in the dark and germination success was recorded after seven days of incubation. Cyst formation was triggered by the following treatments: Nitrogen and phosphorus limitation for single strains (a, b, c) and the combination of five strains (mix) and a drop of temperature for the combination of five strains (T mix).

III

Received: 10 August 2018 | Revised: 30 January 2019 | Accepted: 5 February 2019

DOI: 10.1002/ece3.5009

ORIGINAL RESEARCH

WILEY Ecology and Evolution Open Access

Future temperature and salinity do not exert selection pressure on cyst germination of a toxic phytoplankton species

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Funding information
Strategic Research Environment ECOCHANGE; Svenska Forskningsrådet Formas; Suomen Akatemia, Grant/Award Number: 310449; Walter ja Andrée de Nottbeckin Säätiö; Tryggers research Foundation; Marie Skłodowska-Curie grant, Grant/Award Number: 659453

Abstract

Environmental conditions regulate the germination of phytoplankton resting stages. While some factors lead to synchronous germination, others stimulate germination of only a small fraction of the resting stages. This suggests that habitat filters may act on the germination level and thus affect selection of blooming strains. Benthic "seed banks" of the toxic dinoflagellate *Alexandrium ostenfeldii* from the Baltic Sea are genetically and phenotypically diverse, indicating a high potential for adaptation by selection on standing genetic variation. Here, we experimentally tested the role of climate-related salinity and temperature as selection filters during germination and subsequent establishment of *A. ostenfeldii* strains. A representative resting cyst population was isolated from sediment samples, and germination and reciprocal transplantation experiments were carried out, including four treatments: Average present day germination conditions and three potential future conditions: high temperature, low salinity, and high temperature in combination with low salinity. We found that the final germination success of *A. ostenfeldii* resting cysts was unaffected by temperature and salinity in the range tested. A high germination success of more than 80% in all treatments indicates that strains are not selected by temperature and salinity during germination, but selection becomes more important shortly after germination, in the vegetative stage of the life cycle. Moreover, strains were not adapted to germination conditions. Instead, highly plastic responses occurred after transplantation and significantly higher growth rates were observed at higher temperature. High variability of strain-specific responses has probably masked the overall effect of the treatments, highlighting the importance of testing the effect of environmental factors on many strains. It is likely that *A. ostenfeldii* populations can persist in the future, because suitable strains, which are able to germinate and grow well at potential future climate conditions, are part of the highly diverse cyst population.

KEYWORDS
adaptation, *Alexandrium ostenfeldii*, climate change, dinoflagellates, excystment, resting stage

1 | INTRODUCTION

In temporally variable environments, the formation of dormant resting stages (propagules) is an effective strategy to secure survival of individual organisms (Lennon & Jones, 2011; Marcus, 1998) and protect populations against short- and long-term environmental fluctuations (Ellegaard & Ribeiro, 2018; Morris et al., 2008). Dormant propagule banks integrate genetically and physiologically varying individuals produced in different seasons and years and form genetic reservoirs that exceed the diversity of active populations (Brendonck & De Meester, 2003). Propagule banks are thus an important factor defining the potential of organisms and populations to persist through environmental change.

Many phytoplankton species form dormant resting stages to survive unfavorable conditions (Fryxell, 1983). Dinoflagellates, a group of phytoplankton that can cause harmful algal blooms, produce fossilizable resting cysts (Dale, 1983). These can survive in sediments of lakes and oceans for many years and even decades (Ribeiro et al., 2011). Their germination may be endogenously controlled (Fischer et al., 2018), but typically defined environmental settings, often representative of suitable growth conditions for the respective species (Rengefors & Anderson, 1998) trigger excystment. Germination may be highly synchronized, leading to excystment of the entire viable seed pool once the right trigger is present (Anderson & Rengefors, 2006; Genovesi et al., 2009). Alternatively, only fractions of the cyst pool may germinate at specific conditions along an environmental gradient (Anglès, Garcés, Reñé, & Sampedro, 2012; Kim, Park, & Han, 2002; Moore et al., 2015) suggesting that cysts with diverse germination requirements are present in the sediment. At the same time, high genetic and phenotypic diversity has been observed in a dinoflagellate cyst pool from Baltic Sea sediments (Kremp et al., 2016).

Selection from standing genetic variation is an important mechanism of evolutionary adaptation whereby a population develops toward a phenotype that best fits the present environment (Orr, 2005). The ability to adapt is an important prerequisite to persist under stressful conditions or realize ecological opportunities arising from climate change (Hoffmann & Sgrò, 2011). The co-occurrence of different phenotypic or adaptive traits in a population allows for natural selection of the best suitable genotype under certain environmental conditions. Studies on plants provide evidence that environmental factors exert natural selection on germination (Donohue et al., 2010). Selection for increased fecundity, for example, favors early germination, and selection on mortality can favor either early or delayed germination, depending on when a mortality event occurs (Donohue et al., 2010). The environment can thus act as a filter, which only allows individuals with a particular trait or phenotype to establish and persist, excluding all others (Kraft et al., 2015). Such environmental filters were shown to prevent germination of plant seeds in the season of highest threat for seedling establishment (Fernández-Pascual et al., 2017). It is likely that also the trait-dependent release of phytoplankton into the water column is regulated by respective environmental filters that prevent germination of genotypes which cannot adjust to unsuitable growth conditions.

Here, we investigate this hypothesis using the dinoflagellate *Alexandrium ostenfeldii* (Paulsen) Balech and Tangen, which forms toxic blooms in shallow coastal waters of the Baltic Sea, as a model species. Like many dinoflagellates from seasonal environments, *A. ostenfeldii* produces resting cysts as part of its life cycle and the cyst pool in coastal sediments was found to be genetically diverse (Tahvanainen et al., 2012). At the same time, considerable variation in temperature and salinity-related traits occurred among individuals recruited from respective cysts (Kremp et al., 2016; Suikkanen, et al., 2013) which was suggested to serve adaptation of *A. ostenfeldii* populations to changing climate conditions in the Baltic Sea (Kremp et al., 2016).

The Baltic Sea is one of the largest brackish water bodies in the world, with a salinity gradient ranging from around 30 to 1 (BACC, 2008; Meier et al., 2006). Due to global warming an overall reduction of sea surface salinity by 1 to 3 psu, depending on the geographic region, is expected in the future (Meier et al., 2012, 2006). Besides salinity, temperature is a key environmental factor and for the total basin of the Baltic Sea a mean annual temperature increase of 3 to 5°C has been suggested for the late 21st century (Graham et al., 2008). This increase might be even more pronounced in stratified shallow coastal embayments of the Baltic Sea, which are a preferred habitat of *A. ostenfeldii*. Increasing summer temperatures may aid expansion of this species in the future (Kremp et al., 2012), which could be problematic due to its ability to produce several potent toxins (Martens et al., 2016; Salgado et al., 2015; Van de Waal et al., 2015), with demonstrated effects, like rapid behavioral disturbance and incapacitation, on co-occurring biota (Sopanen et al., 2011). A thorough understanding of the mechanisms regulating the response of the species to the predicted changes is crucial to forecast its impact under future climate conditions.

In this study, we experimentally tested the role of climate-related salinity and temperature changes as selection filters during germination and subsequent establishment of *A. ostenfeldii* strains. We were aiming to define if certain strains from the diverse cyst pool are selected by future temperature and salinity conditions and if selection acts at the level of cyst germination or later in the growth phase of the life cycle. Additionally, we investigated if strains which germinated at a specific temperature or salinity are adapted to those conditions or if their phenotypic response depends on the conditions after germination. We hypothesized that (a) temperature and salinity affect the final germination success (i.e., the maximum germination success at a given condition after four weeks) of *A. ostenfeldii* resting cysts and (b) successfully germinated strains are adapted to respective temperature and salinity conditions and will grow best at the same conditions. To test our hypotheses, germination experiments were carried out with wild resting cysts, isolated from sediments in a well-described northern Baltic bloom site, and subsequent growth of resulting strains was recorded. Reciprocal transplantation experiments were carried out with successfully germinated strains to assess if *A. ostenfeldii* genotypes are adapted to germination

conditions and therefore selected, or if they can acclimate to new conditions rapidly.

2 | MATERIAL AND METHODS

We have strong support from amplified fragment length polymorphism and microsatellite data, showing that 95%–100% of Baltic Sea *A. ostenfeldii* strains established from a single resting cyst are unique (Tahvanainen et al., 2012, J. Jerney et al., unpublished), to assume that strains represent individual genotypes. Therefore, the terms "strain" and "genotype" are used synonymously in this study.

2.1 | Sampling

Sediment samples were collected in September 2015 from a shallow embayment in the Föglö archipelago, Åland, Northern Baltic Sea (60°05, 6′N, 20°32, 4′E) which has been described in detail earlier (Hakanen et al., 2012; Kremp et al., 2009) and corresponds to station 4 in the latter reference. Sediment cores were taken from the very shallow bay (water depth <3 m) with a gravity corer (Limnos, Turku), and the uppermost flocculent sediment layer was transferred to 50 ml centrifugation tubes (Falcon). The tubes were entirely filled up with sediment and stored at cold (4°C) and dark conditions until further use to prevent germination of resting cysts before starting the experiments.

2.2 | Sediment processing

To detach resting cysts from sediment particles, subsamples were sonicated prior to the experiments for 30 s on constant duty cycle with a frequency of 20 kHz (Brandelin Sonoplus sonicator HD 2200). During sonication, the samples were cooled with ice to avoid temperature increase and sieved afterward to isolate the 30–76 µm fraction, containing *A. ostenfeldii* resting cysts. The material retained on the 30 µm screen was transferred into a 15 ml polypropylene centrifuge tube (Falcon) and diluted with filtered seawater (6 psu) to obtain a cyst slurry for microscopic isolation of single cysts (Figure 1).

2.3 | Germination success

To determine if temperature or salinity affect the germination rate, a first germination experiment was carried out in March 2016. For this purpose, subsamples of the cyst slurry were inspected microscopically (Leica DMI3000 B inverted microscope) to obtain *A. ostenfeldii* cysts. Single resting cysts were isolated with a glass micropipette and transferred to 24-well plates, each well filled with 2 ml of f/2-Si culture medium (Guillard, 1975; Guillard & Ryther, 1962) prepared from filtered (0.2 µm), autoclaved Baltic Sea water. For each treatment, three replicates, with 10 cysts each, were used. Isolated cysts were incubated at the following conditions: Control (C) 16°C, 6 psu, (representing average germination conditions in the Baltic Sea); high temperature (T) 20°C, 6 psu; low salinity (S) 16°C, 3 psu; and high temperature combined with low salinity (TS) 20°C, 3 psu. Cysts were incubated at 14:10 hr light:dark cycle; and a light intensity of ~100 µmol photons m^{-2} s^{-1}. Germination success was recorded every 2–3 days microscopically, during a period of 26 days, by counting the number of empty versus full cysts on the bottom of each well. Germination was evaluated as successful if an empty cyst wall was found and unsuccessful if the cyst remained intact and did not germinate after the incubation period. Germination success was calculated as number of germinated cysts relative to the total number of cysts per well and plotted over time.

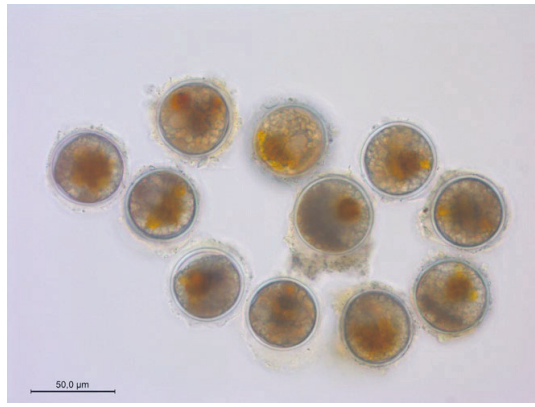

FIGURE 1 Typical resting cysts of the toxin producing marine dinoflagellate *Alexandrium ostenfeldii*, isolated from sediment sampled at the Åland Islands in the Baltic Sea

2.4 | Selection at germination level

The purpose of our selection experiment (Figure 2a) was to find out if the final germination success is affected by different temperature and salinity scenarios and to identify where selection happens: during germination or later in the vegetative stage of the life cycle (defined by the amount of cells established per cyst). Here, the same treatments were used as for the first germination experiment, but this time 96 cysts were isolated per treatment and placed individually in wells of 24-well plates, each well containing 2 ml of f/2-Si medium. Two weeks after start of the experiment, germination success of each cyst was recorded microscopically (Zeiss Stemi SV 11 stereo microscope and Leica DMI3000 B inverted microscope). Germination was evaluated as successful if at least one empty cyst and vegetative cells or several immobile cells were present and unsuccessful if one full cyst was present. Additionally, the number of vegetative cells (originating from one cyst) was estimated for each well to assess how vegetative growth is affected immediately after germination and grouped in categories: <10, 11–50, 51–100, and >100 cells. Four weeks after the start of the experiment, cysts, which were not germinated yet, were re-inspected and the germination success recorded. The response of the strains within these first four weeks after germination is unaffected by any further processing

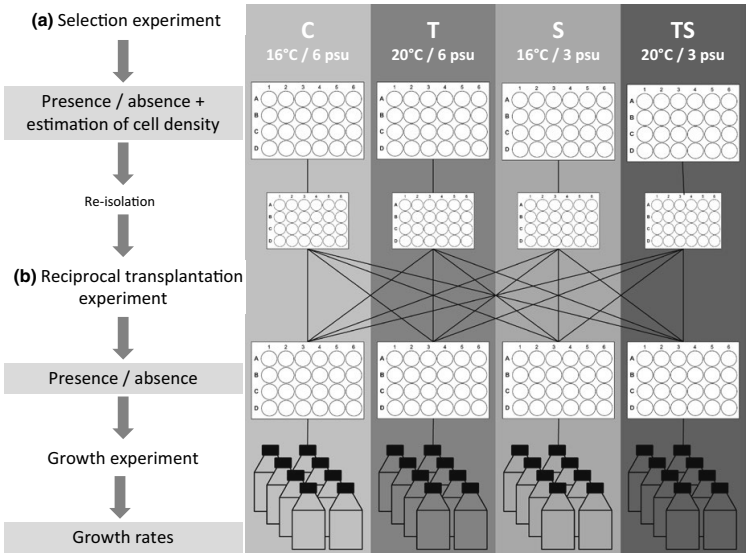

FIGURE 2 Setup of the main experimental series: (a) Germination experiment to define the life stage susceptible to selection, (b) reciprocal transplantation and growth experiments to assess if *A. ostenfeldii* is adapted to specific germination conditions. Capital white letters indicate the treatment

steps, like re-isolation of single cells and transfer of cultures to larger containers, which can affect survival of some strains, and therefore considered relevant.

2.5 | Adaptation to germination conditions

Clonal cultures established from successfully germinated resting cysts of the selection experiment were used to perform reciprocal transplantation experiments (Figure 2b). The aim of these experiments was to test whether strains which had germinated under specific conditions of the selection experiment (C, S, T, and TS) were adapted to those conditions. If the strains were adapted to their germination conditions, one would expect them to grow best at the same conditions. Transplantation to other conditions, in contrast, would result in lower growth rates. Higher growth rates after transplantation would indicate that strains are not adapted to their germination conditions, but they can acclimate to different conditions rapidly and proof at the same time that selection does not happen during germination. For this purpose, eight well-growing cultures of each treatment of the germination experiment were randomly selected. Single cells were re-isolated after 2 weeks (after 4–5 divisions) to establish clonal cultures, followed by a short culturing period of 8 weeks (around 10 divisions) under the same conditions, to increase the cell number for the transplantation experiment. The culturing period was kept as short as possible to minimize a potential acclimation to the respective growth conditions. After the short culturing period, single vegetative cells were transplanted reciprocally from respective germination conditions to all other conditions. Strains which were transplanted to the same growth conditions as their initial germination conditions (from C to C, S to S, T to T or TS to TS) served as controls in this experiment. For phenotypic characterization of the transplanted strains, growth experiments were carried out in tissue culture flasks, with a starting concentration of 500 cells per ml and a total volume of 40 ml. Growth was inferred from the development of Chl *a* fluorescence in each flask. Samples were measured at the start of the experiment, 7 days later and every 3–4 days for a period of one month after that with a fluorescence spectrophotometer (Varian Cary Eclipse; excitation 440 nm, emission 680 nm) equipped with a well plate reader. Growth rates (*r*, rate of increase) were calculated based on the longest period of exponential growth using the equation $r = \ln(N_t/N_0)/\Delta t$ (Wood et al., 2005). The interval of exponential growth was determined from growth curves established for each experimental culture and included at least three time points.

2.6 | Statistical analysis

All statistical analyses were performed with R version 3.4.4 (R Core Team, 2018) and RStudio (RStudio Team, 2015). For the selection experiment, a Pearson's Chi-squared test was performed to check if the treatment has an effect on the ratio of germinated versus not germinated cysts. A simple linear regression was applied to model the estimated abundance of vegetative cells after germination as function of the treatment, followed by an analysis of variance (ANOVA). The variate "treatment" was categorical with four levels (C, S, T, and TS).

For the statistical analysis of the transplant experiment data, we distinguished between two effects: (1) if the growth conditions after transplantation were the same as before (coded as 0) or different (coded as (1) and (2) if conditions after transplantation were different from the germination condition, which other treatment (C, S, T, or TS) was applied. Same conditions before and after transplantation served as controls (e.g., transfer from C to C, S to S, T to T or ST to ST). A linear regression was used to model the

growth rate after transplantation, followed by a simple two-way ANOVA with two categorical predictors (2 levels and 4 levels) and the interaction between them. The strain could not be included as a random effect, because our experimental setup only included one observation per strain for each treatment combination, which made it impossible to separate a treatment effect from the strain effect. Model assumptions were verified by plotting residuals versus fitted values.

3 | RESULTS

3.1 | Germination success

During the first germination experiment, cysts of *A. ostenfeldii* germinated rapidly after exposure to favorable conditions (Figure 3). Regardless of the treatment, more than 75% of the cysts germinated within 8 days. The fastest germination was observed in the T and TS treatment, where cells occurred already one day after setting up the experiment, and lower salinity (S) resulted in slightly delayed germination (starting at day 3). The final germination success ranged from 80%–100% after 25 days.

3.2 | Selection at germination level

In our selection experiment, more than 84% of the cysts had germinated after two weeks of incubation, regardless of the treatment (Figure 4). The highest germination success was observed for the control, reaching 92%, followed by treatment T, TS, and S with 88%, 86%, and 84%, respectively. We found no significant difference between the ratio of germinated and not germinated cysts between the treatments ($\chi^2 = 3.27$, $df = 3$, $p = 0.35$). When modeling the estimated abundance of vegetative cells after germination as function of the treatment, a significant effect of the treatment was found (ANOVA: $F_{3,328} = 86.74$, $p < 0.001$) with an R^2 of 0.44. Estimation of the cell numbers established per resting cyst revealed that high temperature (T) supported significantly higher cell numbers, compared to the control, ($t = -8.07$; $df = 328$, $p < 0.001$) whereas low salinity (S) resulted in significantly lower cell numbers, compared to the control ($t = -8.31$; $df = 328$, $p < 0.001$) (Figure 4).

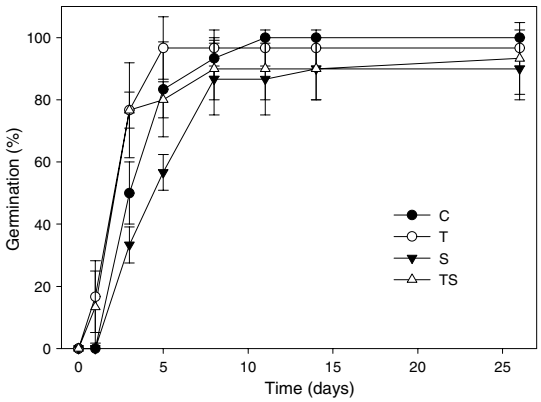

FIGURE 3 Cumulative percentage of *A. ostenfeldii* resting cysts, germinated under different experimental conditions: 16°C, 6 psu (C); 20°C, 6 psu (T); 16°C, 3 psu (S); 20°C, 3 psu (TS). Three replicates per treatment with 10 cysts in each (means ± standard deviation, $n = 3$)

3.3 | Adaptation to germination conditions

To test if germinated *A. ostenfeldii* strains from the selection experiment (Figures 2a and 4) are adapted to their germination conditions, a subset of these strains were used to perform a reciprocal transplantation experiment (Figures 2b and 5). Overall, growth rates were affected by growth rather than germination conditions. Transplantation to higher temperature resulted in higher growth rates for most strains, and also the maximal growth rates were observed after transplantation to T, at all germination conditions. When modeling the growth rate as function of the germination condition in combination with the growth condition, a significant effect of the growth condition was found (two-way ANOVA, $F_{7,118} = 11.87$, $p < 0.001$). Additionally, growth rates of strains growing at condition T were significantly different from the control ($t = 3.28$; $df = 118$, $p < 0.01$). The germination condition, as well as the interaction between different germination and growth condition, had no significant effect on the growth rates. Strains with the same germination and growth conditions (e.g., transplantation from C to C) served as a control in this experiment and the control of treatment C (16°C and 6 psu) had an average growth rate of 0.09/day. After transfer from C to S, high response variability was observed. The majority (62.5%) of the strains did not grow, and

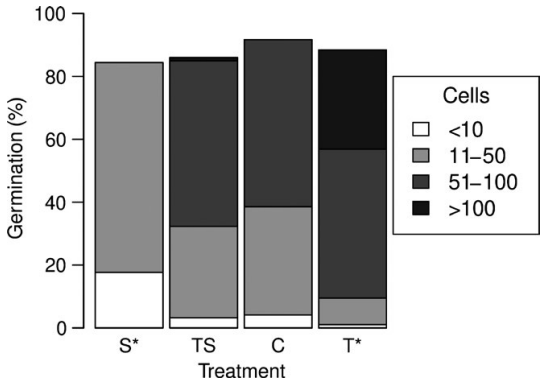

FIGURE 4 Percentage of *A. ostenfeldii* resting cysts germinated, relative to the total amount of cysts isolated for each treatment, two weeks after incubation. Control (C): 16°C, 6 psu, $n = 94$; high temperature (T): 20°C, 6 psu, $n = 95$; low salinity (S): 16°C, 3 psu, $n = 96$; high temperature combined with low salinity (TS): 20°C, 3 psu, $n = 93$. Colors of the stacked bars represent the estimated abundances of vegetative cells originating from each cyst grouped in categories: <10 cells, 11–50 cells, 51–100 cells, and >100 cells. Asterisks indicate treatments significantly different from the control with respect to the cell numbers ($p < 0.001$)

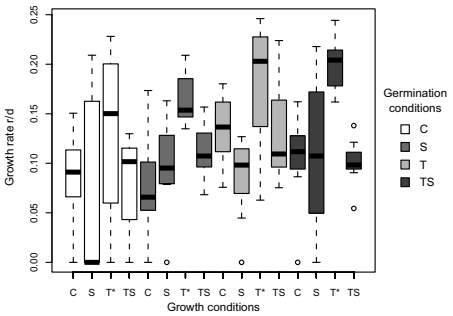

FIGURE 5 Box-and-whisker plots showing growth rates of strains transplanted from germination conditions (indicated by the color of the bars) reciprocally to the growth conditions. Control (C): 16°C, 6 psu; high temperature (T): 20°C, 6 psu; low salinity (S): 16°C, 3 psu; high temperature combined with low salinity (TS): 20°C, 3 psu; $n = 8$ for all treatments (except for T and TS germinated under condition TS, $n = 7$). Asterisks indicate treatments significantly different from the control ($p < 0.05$). The plots include the lower and upper extremes (horizontal lines outside the box), 25th and 75th percentiles (top and bottom frame of the box) and the median (solid line within each box)

among the growing strains, 25% exceeded the maximum growth rate of the control. Similarly, transplantation from C to T resulted in highly variable growth rates, but with higher mean growth rate (0.13/day). Transplantation from C to TS did not alter growth rates substantially but resulted in a similar mean growth rate as the reference (0.08/day). The growth rates of the reference S (mean 0.10/day) were comparable to C, but showed less variation. When transplanted from S to C the mean growth rate dropped to 0.08/day and greater variation occurred. After transplantation from S to T and TS, growth rates increased on average to 0.16 and 0.11/day, respectively. For strains germinated at condition T, different responses to transplantation were observed. We measured the highest growth rates (mean 0.18/day) for the control and transplantation to all other treatments led to reduced growth. Different responses to transplantation were also recorded for strains germinated at condition TS. Transfer from TS to C did not alter the growth rates substantially (mean 0.10/day). Transplantation from TS to S resulted in a highly variable response, which is comparable to the transplantation from C to S and TS to T caused the highest mean growth rates of all treatments (0.20/day).

4 | DISCUSSION

We were aiming to define if certain strains from the diverse *A. ostenfeldii* cyst pool are selected by future temperature and salinity conditions and if selection acts at the level of cyst germination or later in the active phase of the life cycle. Additionally, we investigated if strains which germinated at a specific temperature or salinity are adapted to those conditions or if their phenotypic response depends on the conditions after germination. We hypothesized that (a) temperature and salinity affect the final germination success of *A. ostenfeldii* cysts and (b) successfully germinated strains are adapted to respective temperature and salinity conditions. Contrary to our hypothesis, the majority of *A. ostenfeldii* resting cysts, isolated randomly from the seed bank of Baltic surface sediments, are able to germinate within a short time after exposure to all tested conditions. Both factors, however, affected growth after germination significantly. The higher relevance of conditions for growth compared to germination was emphasized by the results of the transplant experiments where growth rates of reciprocally transplanted strains were a response to the growth conditions after transplantation, rather than their germination conditions. Higher temperature caused significantly higher growth rates, and in addition, we observed strong variation in phenotypic responses (growth) after transplantation.

4.1 | Selection at germination level

In contrast to our expectation, there was no significant influence of the tested salinities and temperatures on germination rate, which was over 75% at all conditions. Changes of these environmental factors in the tested ranges thus do not seem to act as filters, preventing germination of unsuited strains. Slightly delayed germination at lower salinity and quicker germination at high temperature suggest that there is a weak effect of both environmental factors on germination (Figure 3). It led to a time difference of approximately five days between the maximum numbers of cysts germinated at respective treatments. This time span is very short in relation to the long growth season of *A. ostenfeldii* (Hakanen et al., 2012); therefore, we do not consider these effects relevant for the composition of the populations initiating bloom formation in nature. Our results are comparable to other studies on germination behavior of dinoflagellates, showing that environmental factors can slow down or accelerate germination, without affecting the final germination success (Binder & Anderson, 1987; Blanco et al., 2009; Genovesi et al., 2009; Moore et al., 2015). Similar germination behavior is well documented for plants, where suboptimal temperature can lead to a slower germination rate, but still allow successful germination (Bewley & Black, 1982). In contrast, environmental factors can represent strong germination filters, allowing for natural selection in plants (Donohue et al., 2010). Natural selection on germination timing of *Arabidopsis thaliana* was, for example, suggested to be an efficient sieve that can determine which genotypes can persist in different geographic locations (Donohue et al., 2005). The relevance of temperature as environmental filter for germination was recognized for several cold-water dinoflagellates (Kremp & Anderson, 2000; Rengefors & Anderson, 1998), but temperature and salinity limits for germination of Baltic *A. ostenfeldii* have not been defined yet. We know that at least temperature becomes selective at extreme values (Jerney et al., unpublished data), which might be also true for salinity, but not in the relatively narrow range tested in our study. If germination of *A. ostenfeldii* resting stages remains unaffected by the tested temperature and salinity conditions, a large fraction of the seed bank could potentially germinate as soon as conditions become suitable.

In unpredictable environments, the prevention of germination of some seeds through dormancy was suggested to reduce the risk of extinction if conditions were to turn unfavorable after germination (Donohue et al., 2010). We assume that Baltic *A. ostenfeldii* populations do not exhibit a strongly pronounced dormancy period, since cysts are able to germinate throughout the year at a high rate (Jerney et al., unpublished data). A more realistic scenario is that under suitable conditions indeed a large part of the population germinates and is able to tolerate a broad range of environmental conditions thereafter. In addition, vegetative cells may form temporary resting cysts to escape unsuitable conditions again, as frequently observed in cultures.

4.2 | Postgermination selection

In contrast to our first hypothesis, the environmental factors tested, herein, did not select for certain strains during germination, but became relevant during the growth phase, shortly after germination. The observed effects of temperature and salinity on growth of *A. ostenfeldii* in our study are comparable to earlier findings (Kremp et al., 2012, 2009; Suikkanen et al., 2013) and underpin our assumption that temperature and salinity exert selection pressure on the growing population. The direct response to selection depends on the level of standing genetic variation in a population (Bell & Collins, 2008) and by supporting or suppressing growth of certain strains temperature and salinity have the potential to affect the genetic composition of future populations. Baltic *A. ostenfeldii* populations seem to be geno- and phenotypically diverse (Kremp et al., 2016), suggesting that suitable individuals can be selected under environmental change.

We found that reduced salinity was unfavorable and higher temperature beneficial for many strains, but in our TS treatment, the positive effect of higher temperature was apparently compensated by lower salinity, resulting in growth rates similar to the control. The two selected, climate change-related parameters have apparently opposite effects on growth, which highlights the importance of studying the effect of multiple environmental factors simultaneously, since they may result in a different response, compared to single factors. Although the TS treatment, as the most likely for future conditions, did not result in the highest growth rates, in most cases it was very comparable to the control (present conditions), indicating that *A. ostenfeldii* seems to be "well-prepared" for the future. As shown by our experiments, suitable strains exist and there should be enough genetic variance in the population (Kremp et al., 2016) to support adaptation.

4.3 | Acclimation and trait variability

Our second hypothesis was that successfully germinated strains are adapted to respective temperature and salinity conditions and will grow best at those conditions. In contrast to our expectations, successfully germinated strains were not adapted to germination conditions. Instead, they were able to adjust to temperature and salinity different from their germination conditions and even outperform nontransplanted control strains. This indicates that *A. ostenfeldii* has a broad germination and growth tolerance for the tested range of temperature and salinity and a high acclimation potential, which is common for phytoplankton living in fluctuating ecosystems (Collins et al., 2013). Offspring will most likely encounter conditions different from its parents, but will adjust to them by phenotypic plasticity (Bell & Collins, 2008). The lack of adaptation to temperature and salinity conditions found in our study seems to contradict earlier observations, which proposed that different responses to temperature and salinity changes reflect adaptations to respective conditions (Kremp et al., 2016).

Moreover, we observed high growth rate variability after transplantation, when comparing growth rates within each set of transplanted strains, showing that responses to temperature and salinity changes can be strain specific. High strain-specific trait—or phenotypic—variability has been demonstrated for this species before (Kremp et al., 2016; Suikkanen et al., 2013) and underlines how crucial it is to test responses to environmental conditions with a large number of strains. We used several strains for our experiments, and effects of our treatments have probably been masked by high-trait variability. High intraspecific trait variation has recently been linked to fluctuating selection pressures (Brandenburg et al., 2018) and might partly explain this species' success in shallow waters of the Baltic Sea. The high-trait variability makes it difficult to assess the overall effect of environmental factors on the population, but having a high variability of responses seems be part of the generalist life strategy of this species and the additive genetic variance may often be sufficient to support adaptation to rapid environmental change (Bell & Collins, 2008).

5 | CONCLUSIONS

Germination success of *A. ostenfeldii* resting stages remained unaffected by temperature and salinity, indicating that these factors, in the tested range, do not filter suitable individuals through prevention of germination. However, both parameters exert selection pressure on the vegetative population and thus have the capability to shape the phenotype composition of populations. It is likely that *A. ostenfeldii* populations can persist under predicted future climate conditions (warmer Baltic Sea with lower salinity), due to its flexible germination behavior, high plasticity, and a great adaptation potential, based on large geno- and phenotypic diversity.

ACKNOWLEDGMENTS

This work was funded in part by grants from the Academy of Finland (grant 310449 to J. Jerney, S. Suikkanen, and A. Kremp) and the Walter and Andree de Nottbeck Foundation (J. Jerney). We thank Johan Franzén and Tobias Lipsewers for assistance during sampling, Johanna Oja for helping with the culture work, and Riina Klais for her advice on the statistical analysis. We are grateful for support from the Strategic

Research Environment ECOCHANGE, Swedish Research Council Formas, the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement 659453 and Tryggers research Foundation.

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

JJ, AK, and SS conceived the study. JJ collected the samples. JJ, AK, SS, and EL carried out experimental work and supported data collection. JJ carried out data analysis. JJ and AK lead the writing of the manuscript with input from all authors.

DATA ACCESSIBILITY

Germination and growth rate data can be accessed at Dryad.

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OPEN RESEARCH BADGES

This article has earned an Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at https://doi.org/10.5061/dryad.c8c83nr.

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How to cite this article: Jerney J, Suikkanen S, Lindehoff E, Kremp A. Future temperature and salinity do not exert selection pressure on cyst germination of a toxic phytoplankton species. *Ecol Evol*. 2019;00:1–9. https://doi.org/10.1002/ece3.5009

IV

Harmful Algae 84 (2019) 64–74

Contents lists available at ScienceDirect

Harmful Algae

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Genetic relatedness of a new Japanese isolates of *Alexandrium ostenfeldii* bloom population with global isolates

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ARTICLE INFO

Keywords:
Alexandrium ostenfeldii
Western Japan
Northern Japan
Baltic Sea
Phylogenetic analysis
Genotyping

ABSTRACT

In recent years, blooms of toxic *Alexandrium ostenfeldii* strains have been reported from around the world. In 2013, the species formed a red tide in a shallow lagoon in western Japan, which was the first report of the species in the area. To investigate the genetic relatedness of Japanese *A. ostenfeldii* and global isolates, the full-length SSU, ITS and LSU sequences were determined, and phylogenetic analyses were conducted for isolates from western and northern Japan and from the Baltic Sea. Genotyping and microsatellite sequence comparison were performed to estimate the divergence and connectivity between the populations from western Japan and the Baltic Sea. In all phylogenetic analyses, the isolates from western Japan grouped together with global isolates from shallow and low saline areas, such as the Baltic Sea, estuaries on the east coast of U.S.A. and from the Bohai Sea, China. In contrast, the isolates from northern Japan formed a well-supported separate group in the ITS and LSU phylogenies, indicating differentiation between the Japanese populations. This was further supported by the notable differentiation between the sequences of western and northern Japanese isolates, whereas the lowest differentiation was found between the western Japanese and Chinese isolates. Microsatellite genotyping revealed low genetic diversity in the western Japanese population, possibly explained by a recent introduction to the lagoon from where it was detected. The red tide recorded in the shallow lagoon followed notable changes in the salinity of the waterbody and phytoplankton composition, potentially facilitating the bloom of *A. ostenfeldii*.

1. Introduction

About half of the species belonging to the genus *Alexandrium* are recognized as harmful either due to toxin production or mass occurrences related to fish mortality (Anderson et al., 2012). Blooms of the toxic *Alexandrium* species can have a negative influence on higher trophic levels directly (Sopanen et al., 2011) or through toxin accumulation and transfer in the food web (e.g. Jester et al., 2009; Setälä et al., 2014). *Alexandrium ostenfeldii* (Paulsen) Balech and Tangen, 1985, is a globally distributed marine planktonic dinoflagellate (e.g. Mackenzie et al., 1996; John et al., 2003; Gribble et al., 2005; Kaga et al., 2006; Kremp et al., 2009; Almandoz et al., 2014; Tillmann et al.,

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https://doi.org/10.1016/j.hal.2019.02.005
Received 28 November 2018; Received in revised form 12 January 2019; Accepted 2 February 2019

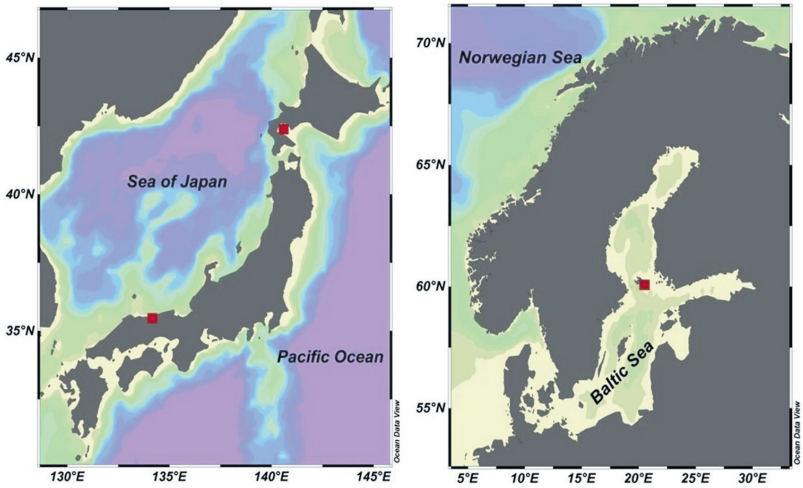

Fig. 1. Sampling locations indicated by squares in western and northern Japan and in the Baltic Sea.

2014), which produces three different types of neurotoxins: paralytic shellfish poisoning toxins, spirolides and gymnodimines (e.g. Cembella et al., 2000; Van de Waal et al., 2015; Harju et al., 2016). The type of toxin produced differs between the strains and their geographic origin (Lim and Ogata, 2005; Kremp et al., 2009; Otero et al., 2010; Touzet et al., 2011; Salgado et al., 2015), and the toxin profile might be genetically determined (Suikkanen et al., 2013). Elevated temperature and pCO_2 levels have been found to influence *A. ostenfeldii* toxin composition and growth rate, which may lead to increased toxic bloom events in the future (Kremp et al., 2012).

Until recently *A. ostenfeldii* was considered to be a background species present in low abundances (e.g. Hansen et al., 1992; John et al., 2003; Gribble et al., 2005), however, dense blooms have been reported from the coastal areas of Finland (Kremp et al., 2009), the Netherlands (Burson et al., 2014), Italy (Ciminiello et al., 2006), and the U.S.A. (Borkman et al., 2012). In the northern Baltic Sea and in a brackish creek in southwestern Netherlands, the blooms of *A. ostenfeldii* have become a reoccurring phenomenon, explained by high sea surface temperatures (> 15 °C), low DIN:DIP ratios, and grazer densities and high cyst abundances (Hakanen et al., 2012; Brandenburg et al., 2017). In Japan, *A. ostenfeldii* (approx. $3*10^5$ cells L^{-1}) first bloomed together with *Levanderina fissa* (approx. $1.246*10^6$ cells L^{-1}) in a shallow semi-enclosed lagoon in autumn 2013. The strains isolated during the bloom produced paralytic shellfish poisoning toxins (H. Oikawa unpubl. data). This was also the first record of the species in western Japan, as *A. ostenfeldii* has been previously present in low abundances (10 - 320 cells L^{-1}) along the Pacific coast of Japan and in the Seto Inland Sea (Kaga et al., 2006; Yuki and Yoshimatsu, 2012).

The species produces resting cysts as a part of its life cycle (Mackenzie et al., 1996). These can accumulate in the sediments and provide an inoculum for blooms (e.g. Genovesi et al., 2009; Anderson et al., 2014; Mardones et al., 2016), while also facilitating species dispersal to new habitats via ships' ballast water (Hallegraeff and Bolch, 1991) and waterfowl (Tesson et al., 2018). However, before blooming, the non-native species have to pass through several phases, i.e. pioneering, persistence, community entry, to achieve colonization and community maintenance at the new locality (Smayda, 2002, 2007). Thus, a sudden bloom might not reflect a recent introduction but rather a change in the environment favoring particular species (Smayda, 2007). This was exemplified by a bloom of *Gymnodinium catenatum* in New Zealand after a La Nina event, which was also the first record of the species in that region, although based on resting cysts, it was already present earlier (Irwin et al., 2003).

In previous phylogenetic studies utilizing various rDNA regions, global *A. ostenfeldii* isolates clustered into six groups representing differences in plate morphology, toxin composition, ecophysiology and geographic distribution (Kremp et al., 2014). The analyses also included strains from northern Japan that grouped separately from other *A. ostenfeldii* isolates (Kremp et al., 2014; Tillmann et al., 2014) and were thus suggested to represent a distinct East Asian genotype (Kremp et al., 2014). This differentiation has been explained by the geographic isolation as the similar pattern was also seen for the isolates from New Zealand (Kremp et al., 2014). At the same time, another *A. ostenfeldii* strain from the Bohai Sea, China, grouped close to the isolates from shallow and mesohaline coastal environments, suggesting a closer relationship with global isolates than with the northern Japanese *A. ostenfeldii* (Gu, 2011; Kremp et al., 2014).

In this study, full-length sequences of the small subunit (SSU), internal transcribed spacer (ITS) and large subunit (LSU) ribosomal DNA regions were obtained from strains originating from western and northern Japan and from the Baltic Sea. The sequences were used for phylogenetic analyses together with *A. ostenfeldii* sequences available in GenBank to further investigate the phylogenetic relationship between Japanese and global *A. ostenfeldii* isolates. In addition, strains from western Japan and from the Baltic Sea were genotyped and the flanking regions as well as the number of microsatellite repeats were compared between both localities to gain further insight into the connectivity between the *A. ostenfeldii* populations from western Japan and the Baltic Sea.

2. Methods

2.1. Study areas and establishment of cultures

The cells used in this study were identified as *A. ostenfeldii* based on the plate morphology, which follows the description by Balech and Tangen (1985). Strains from western Japan were established from vegetative cells isolated during a bloom in autumn 2013 from the Lake Koyama-ike, Tottori Prefecture (35° 29′ 54.7″ N, 134° 09′ 20.2″ E, Fig. 1) with a surface area of 7 km^2 and an average depth of 2.8 m (Nomura et al., 2009). During the time of isolation, the salinity was 11.44, temperature 26.23 °C and Chl *a* 156.78 µg/L. The cells from northern Japan originate from the Funka Bay, Hokkaido (42° 02′ 26.7″ N, 140° 49′ 37.4″ E), that has an average depth of 59 m (Takahashi

et al., 2005) and where *A. ostenfeldii* usually appears during spring (March-May), when the average surface salinity is around 32 (Azumaya et al., 2001). Despite several attempts, it was not possible to establish cultures and thus single cells were used for further study. The single cells from northern Japan were collected during March 2008 and 2009 and sequences from the majority of the isolates have been previously used in a report by Nagai et al. (2010). The strains from the Baltic Sea originate from Föglö archipelago, Åland, Finland (60° 05′ 45.8″ N, 20° 31′ 28.4″ E) from a shallow sound (< 3 m) with summer sea surface salinities around 6-7. The sampling site and establishment of cultures are described in more detail by Kremp et al. (2009) and Hakanen et al. (2012).

2.2. DNA extraction and number of strains used

DNA was extracted from cultures grown in a 25 mL F2 medium (Guillard, 1975) in the exponential phase (strains from Finland and western Japan) and from single cells (northern Japan). The extraction was done by using 5% Chelex buffer as described by Nagai et al. (2012). Eight strains from western Japan, eight single cells from northern Japan and four strains from the Baltic Sea were used to investigate their phylogenetic position in comparison with the global isolates. The strains used for phylogenetic analysis are indicated in Table S1. For genotyping by microsatellites and microsatellite sequence comparison additional 27 strains from western Japan and 40 strains from the Baltic Sea were used (Table S2). Isolates JS2, JS3, and JS5 from western Japan were used for phylogenetic analysis, microsatellite genotyping and microsatellite sequence comparison.

2.3. PCR amplification and sequencing for phylogenetic analyses

To determine the full-length SSU, ITS, and LSU rDNA sequences, forward and reverse primers developed by Adachi et al. (1994); Takano and Horiguchi (2004); Ki and Han (2007) were used as well as primers targeting the end of LSU developed by Nagai et al. (2010). PCR amplification was performed on 20 µL reaction volume consisting of 2 µL of PCR buffer, 2 µL of dNTP, 1.2 µL of $MgSO_4$, 0.4 µL of each primer (50 µM), 0.4 µL of enzyme (KOD-Plus-Ver. 2), 12.1 µL of sterile MQ water and 1.5 µL of DNA. The PCR amplification conditions consisted of one denaturation step for 3 min at 94 °C, followed by 30 cycles of 15 s at 94 °C, 30 s at 55 °C and 40 s at 68 °C. The amplified DNA was directly sequenced in both directions using the BigDye™ Terminator v.3.1 Cycle Sequencing Kit and an ABI 3730xl DNA Analyzer (Applied Biosystems). The sequences were edited manually by using MEGA version X (Kumar et al., 2018) and are available from Genbank (Table S1).

2.4. Microsatellite genotyping and genetic variation

The primer pairs and the PCR conditions used are described by Nagai et al. (2015). All PCR products were electrophoresed on an ABI 3730xl DNA Analyzer (Applied Biosystems). Allele sizes were determined using a 600 LIZ size standard (Applied Biosystems) and GeneMapper ver. 4.0 (ABI). MS tools (Park, 2001) was used to estimate the number of alleles, allelic frequency, and gene diversity (Nei, 1987). Allele numbers at the 10 loci ranged from 2 to 12 with an average of 5.3 and estimates of gene diversity (Nei, 1987) varied between 0.10 and 0.92, suggesting that these microsatellites have a good potential to characterize genetic structure of *A. ostenfeldii* at the population level. From both populations 29 isolates were used for genotyping by microsatellites (Table S2).

2.5. Sequence comparison of microsatellite regions

Two microsatellite loci (*Aosten13* and *Aosten296*, Nagai et al., 2015) were amplified following the PCR conditions given by Nagai et al. (2015). Amplified fragments were cloned into the pGEM-T Easy Vector Systems (Promega) and transformed into *Escherichia coli* following the manufacturer's protocol (Promega, 2010). Microsatellite sequences were determined using the BigDye™ Terminator v.3.1 Cycle Sequencing Kit and an ABI3730xl DNA Analyzer (Applied Biosystems). The sequences were edited manually in MEGA version X (Kumar et al., 2018). The strains used for microsatellite sequence comparisons are shown on Figs. 6 and 7 and in Table S2.

2.6. Phylogenetic tree constructions

The sequences from the SSU region were aligned with 26 *A. ostenfeldii* and 13 sequences from different *Alexandrium* species downloaded from the GenBank (Table S1). For the ITS alignment, 22 *A. ostenfeldii* and 10 other *Alexandrium* species sequences were added (Table S1). For the LSU sequences, two different alignments were made to include as many sequences from different geographic locations as possible (shorter alignment) and to explore phylogenetic relationships by using the full-length LSU sequences (longer alignment). For the shorter LSU alignment, 36 *A. ostenfeldii* sequences and 10 other *Alexandrium* species sequences were downloaded from the GenBank (Table S1). For the full-length alignment, 4 *A. ostenfeldii* and 10 other *Alexandrium* species sequences from the GenBank were added (Table S1). The total length of the alignments used for phylogenetic analysis was 1680 base pairs (bp) (SSU), 546 bp (ITS), 599 bp (LSU short alignment) and 3474 bp (LSU full-length alignment) including insertions/deletions. Phylogenetic trees were constructed by using maximum likelihood analysis in MEGA version X (Kumar et al., 2018). The models used for the analysis were as follows: TN93 + G (SSU, LSU both alignments) and HKY + I (ITS). The models for the SSU and LSU were selected based on the lowest Bayesian Information Criterion scores, whereas for the ITS the models with lowest scores did not show a reasonable phylogeny as *A. ostenfeldii* strains from northern Japan were placed on a higher phylogenetic level than other *Alexandrium* species and other *A. ostenfeldii* strains. Thus, several models were tested and the first model producing a more conventional phylogeny was selected. All sites were used for analyses. Tree topologies were supported by bootstrap values calculated with 100 replicates.

2.7. Modelling the origin of northern Japanese isolates

A high resolution (1/50 degree) ocean model covering all coastal regions around Hokkaido, northern Japan, (Kuroda et al., 2014) was used to investigate the potential origin of *A. ostenfeldii* isolates. Environmental conditions from spring 2008 and 2009 were used in the model as the species was detected from the Funka Bay during those years (K. Baba, personal comm.). To model the potential origin of the vegetative cells a backward particle-tracking model based on LTRANS (Schlag et al., 2008) configured for Regional Ocean Modelling System (Shchepetkin and McWilliams, 2003, 2005) was used. During 2008 and 2009, *A. ostenfeldii* cells were detected at depths of 5 and 30 m, and thus the particles were initially set to those depths within a square area of 0.1° around the mouth of Funka Bay, where *Alexandrium ostenfeldii* vegetative cells were observed. The initial spatial interval between adjacent particles was 0.002°. In total, 9654 particles were tracked backward in time for 2 months from the observed days (i.e., 12 March 2008 and 15 March 2009) by reversing the sign of three-dimensional model velocity. A small constant of horizontal diffusion (1 m^2 s^{-1}) was assumed and vertical diffusion was neglected.

3. Results

3.1. Phylogenetic analyses

The strains from the Baltic Sea and western Japan had identical SSU and ITS sequences within each geographic location. In the LSU sequences, there was one base pair (bp) difference between the strains

Table 1
Comparison of the number of substitutions (S) and insertions/deletions (I/D) between sequences from strains of different geographic origin: BS – Baltic Sea, wJP –western Japan, nJP – northern Japan, BSC – Bohai Sea, China; asterisk indicates comparison between short LSU sequences; only substitutions present in all strains from one geographic location were taken into an account. Four sequences from western Japan, 4 (SSU, ITS) and 8 sequences (LSU) from northern Japan, 2 sequences from the Baltic Sea and 1 sequence from the Bohai Sea were used for the comparisons.

	BS *vs* wJP		wJP *vs* nJP		BS *vs* BSC		wJP *vs* BSC	
	S	I/D	S	I/D	S	I/D	S	I/D
SSU	0	0	3	0	1	0	1	0
ITS	11	0	23	2	10	1	1	1
LSU	7	0	20	0	3*	0*	5*	0*

from the Baltic Sea and western Japan. The strains from northern Japan displayed 3 bp differences in the SSU sequences, no differences between the ITS and 5 bp differences in the LSU sequences. There was a 2 bp difference for the SSU, no differences for the ITS and a 3 bp difference for the LSU region between the northern Japanese strains from different years. Comparison between the sequences of different geographical origin showed the presence of a low number of insertions/deletions in the ITS sequences, but no insertions/deletions were found in the SSU or LSU sequences (Table 1). The number of substitutions was the lowest in the SSU sequences and highest in the ITS sequences. The highest number of substitutions in all amplified rDNA regions was found between the sequences from western and northern Japan, whereas the lowest number of substitutions was present between the sequences from western Japan and from the Bohai Sea, China (Table 1).

For comparisons among strains originating from the Baltic Sea and western Japan, no identical SSU sequences were found using GenBank data, and the sequence similarity was 99% with the most similar sequence (1794/1799 bp for all the strains, *A. ostenfeldii*, AB538439, Funka Bay, northern Japan). No identical SSU sequences were found for the strains from northern Japan, and the highest similarity was 99% (1666/1669 bp for AOFUN0801 and 1667/1669 for AOFUN0802 and AOFUN803) with *A. ostenfeldii* strain KJ362003 from Palamos, Spain. For ITS, the Baltic Sea sequences matched 100% with several other *A. ostenfeldii* sequences from the Baltic Sea, e.g. 519/519 bp, KY783632, Gotland, Sweden. For the western Japanese strains, no identical ITS sequences were found, and the similarity with the most similar sequence was 99% (517/519 bp, *A. ostenfeldii*, JN173268, Bohai Sea, China). No identical ITS sequences were found for strains from northern Japan, and the highest similarity was 96% with several strains previously identified as *A. peruvianum* (now considered as *A. ostenfeldii*), e.g. 497/519 bp for all the strains, JX841267, Spain.

For the full-length LSU, the most similar sequence in GenBank to both Baltic Sea sequences (99%, 3955/4004 bp for BS405 and 3956/4004 bp for BS424) was *A. ostefeldii* sequence from the Funka Bay, northern Japan (AB538439). This was also the most similar LSU sequence for all the sequences from western Japan (99%, 3958/4003 bp). For strains from northern Japan, the most similar sequence was the *A. ostenfeldii* sequence JF521637 from Flodevigen Norway (99%, 3136/3256 bp for AOFUN0801, 3180/3219 bp for AOFUN0901, 3179/3219 bp for AOFUN0902, 3180/3219 bp for AOFUN0903).

In the phylogenetic tree based on SSU sequences, the two strains from the Baltic Sea and four strains from western Japan grouped together with *A. ostenfeldii* strains from different locations in the Baltic Sea, one strain from Palamos, Spain (Mediterranean Sea) and a strain from Bohai Sea, China (Fig. 2). The strains from northern Japan, Hokkaido, formed a separate group from the other *A. ostenfeldii* strains, however this was not supported by a bootstrap value. In a phylogenetic tree based on the ITS sequences, two strains from the Baltic Sea grouped together with the other *A. ostenfeldii* strains from the Baltic Sea, one strain from the Netherlands and one strain from the U.S.A. (Fig. 3). This group also included two strains from the east coast of U.S.A. (then considered as *A. peruvianum*) and the clustering was supported by a high bootstrap value (100%). The *A. ostenfeldii* strains from western Japan formed a separate group, which was close to the *A. ostenfeldii* strain from the Bohai Sea, China. The strains from northern Japan, Hokkaido, formed a separate group from all the other strains, which was also supported by a high bootstrap value (100%).

In the phylogenetic tree based on the LSU full-length alignment (Fig. 4), the strains from the Baltic Sea grouped together with another *A. ostenfeldii* strain from the Baltic Sea and the strains from western Japan formed a separate group, which was not supported by a bootstrap value. The strains from northern Japan formed a separate group supported by a high bootstrap value (93%). The strains within this group also showed differentiation, which was supported by bootstrap values (≥ 66%). The phylogeny based on the LSU short alignment (Suppl. Fig. 1) conforms with the other phylogenies in this study as well as with the concatenated ITS-LSU phylogeny by Kremp, et al. (2014).

3.2. Genotyping by microsatellites and sequence variation in franking regions

From ten microsatellite loci, two loci (*Aosten13* and *Aosten 296*) were successfully amplified for strains from both locations. Both loci were polymorphic in the Baltic Sea strains, whereas in the strains from western Japan, only the locus *Aosten13* was polymorphic. The number of alleles varied from 1 to 6 (Fig. 5) with the average number of alleles for western Japanese strains 1.5 ± 0.71 (S.D.) (n = 29) and for the strains from the Baltic Sea the average was 4 with S.D. of 2.83 (n = 29). The Nei's unbiased genetic diversity (Nei, 1987) was 0.074 for the western Japanese strains (*Aosten13*) and 0.792 (*Aosten13*) and 0.520 (*Aosten296*) for the Baltic Sea strains.

In locus *Aosten13*, the 5′ flanking region was almost identical between the strains from the Baltic Sea and the published microsatellite sequence (AB971375) (Fig. 6). At the same time, sequences of western Japanese strains showed on average 6 substitutions and 2 insertions/deletions in this region compared to strains from the Baltic Sea and the published sequence. The number of microsatellite repeats varied from 3 (2) + 15 to 20 CT repeats in the sequences from the Baltic Sea compared to the number of repeats in the published sequence (3 + 17). The sequences of western Japanese isolates had on average 7 CT repeats and contained up to 17 substitutions and 4 insertions/deletions in the microsatellite region, whereas the sequences from the Baltic Sea did not have any substitutions, although insertions/deletions were present. In the 3′ flanking region, the number of substitutions in the strains from the Baltic Sea varied from 0 to 4, whereas in western Japanese sequences the number of substitutions varied between 5 and 6. No insertions/deletions were present in the 3′ flanking region for all the strains.

In *Aosten296*, the 5′ flanking region contained a number of substitutions between the published sequence and sequences from the Baltic Sea and western Japan as well as within the sequences from the Baltic Sea and western Japan (Fig. 7). In general, two main sequence types were present both within the Baltic Sea and western Japanese sequences. Insertions/deletions were present in 6 bases of the sequences, 4 of them in single sequences. In the microsatellite region, 4 substitutions and 13 insertions/deletions were found. The two sequences from western Japan contained 6 and 8 CG repeats and in the Baltic Sea sequences, the number of repeats ranged from 5 to 8, whereas the published sequence contained 12 CG repeats. In the 3′ flanking region, 2 substitutions were present in sequences from both locations and one substitution was found only from a single sequence from the Baltic Sea (BS4 42). Only 2 insertions/deletions were present in this region.

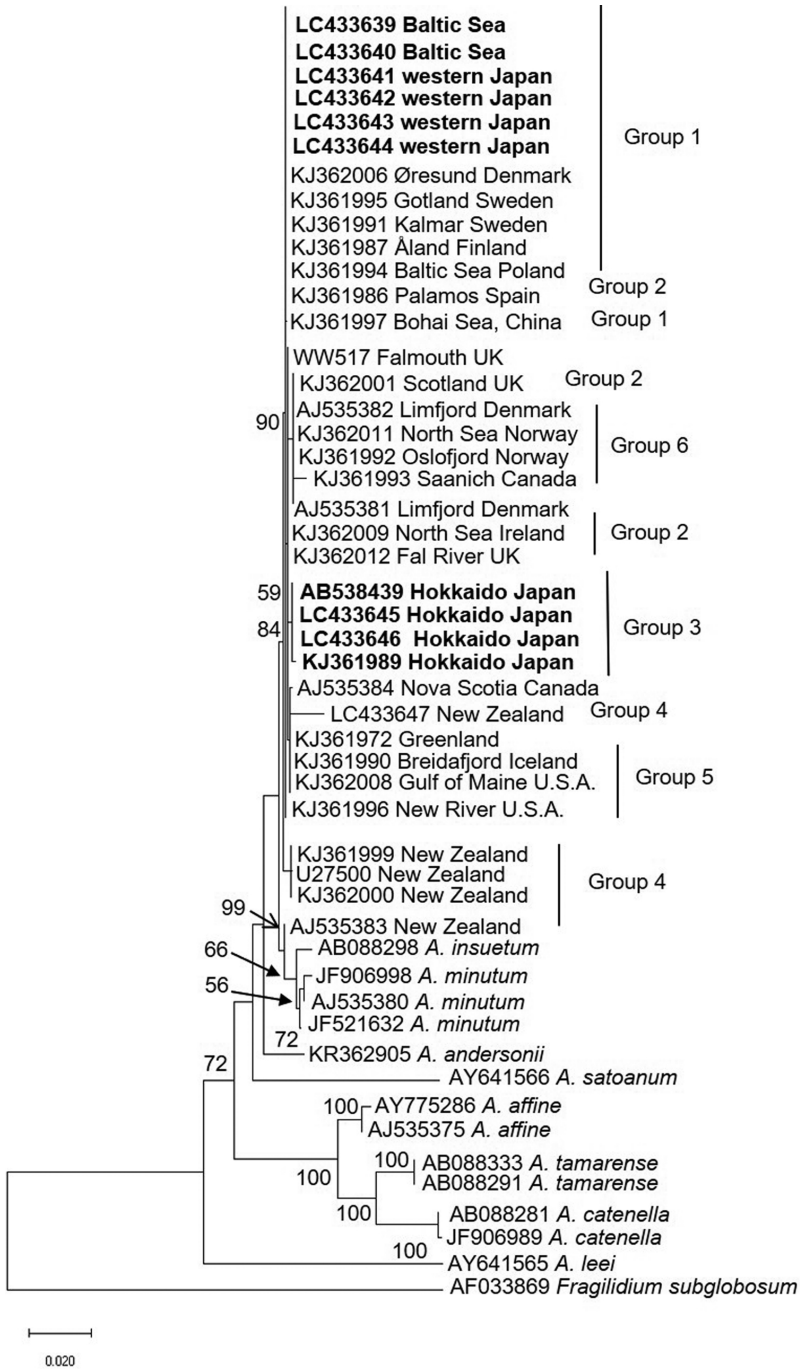

Fig. 2. A maximum-likelihood tree based on the SSU alignment (1679 bp, incl. insertion/deletions) including 50 sequences and *Fragilidium subglobosum* serving as an outgroup. Bootstrap values > 50% are shown. Group assignments based on Kremp et al. (2014).

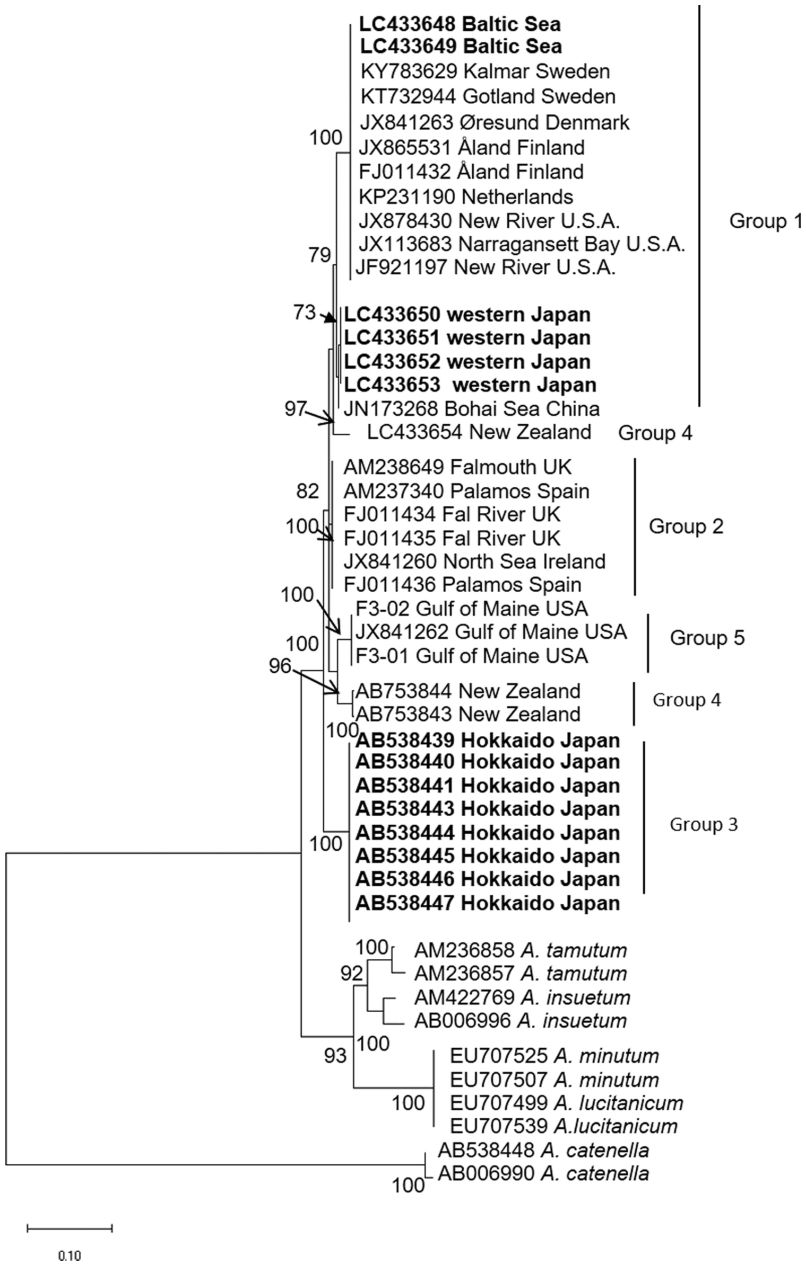

Fig. 3. A maximum-likelihood tree based on the ITS alignment (525 bp, incl. insertion/deletions) including 46 sequences and *Alexandrium catenella* serving as an outgroup. Bootstrap values > 50% are shown. Group assignments based on Kremp et al. (2014).

3.3. Modelled origin of the northern Japanese isolates

In 2008, hydrodynamic particle tracking modeling showed that the majority of the particles observed in the Funka Bay would have been transported there along the north-east coast of Hokkaido, originating from the Sea of Okhotsk or further north along the Pacific coast of the Kurili Islands (Fig. 8). In 2009, most of the particles also originated from the north, transported along the north-eastern coast of Hokkaido (Fig. 8), whereas some particles also originated from the Sea of Japan.

4. Discussion

The phylogenies based on different rDNA regions showed similar genetic grouping as in previous studies by Kremp et al. (2014) and

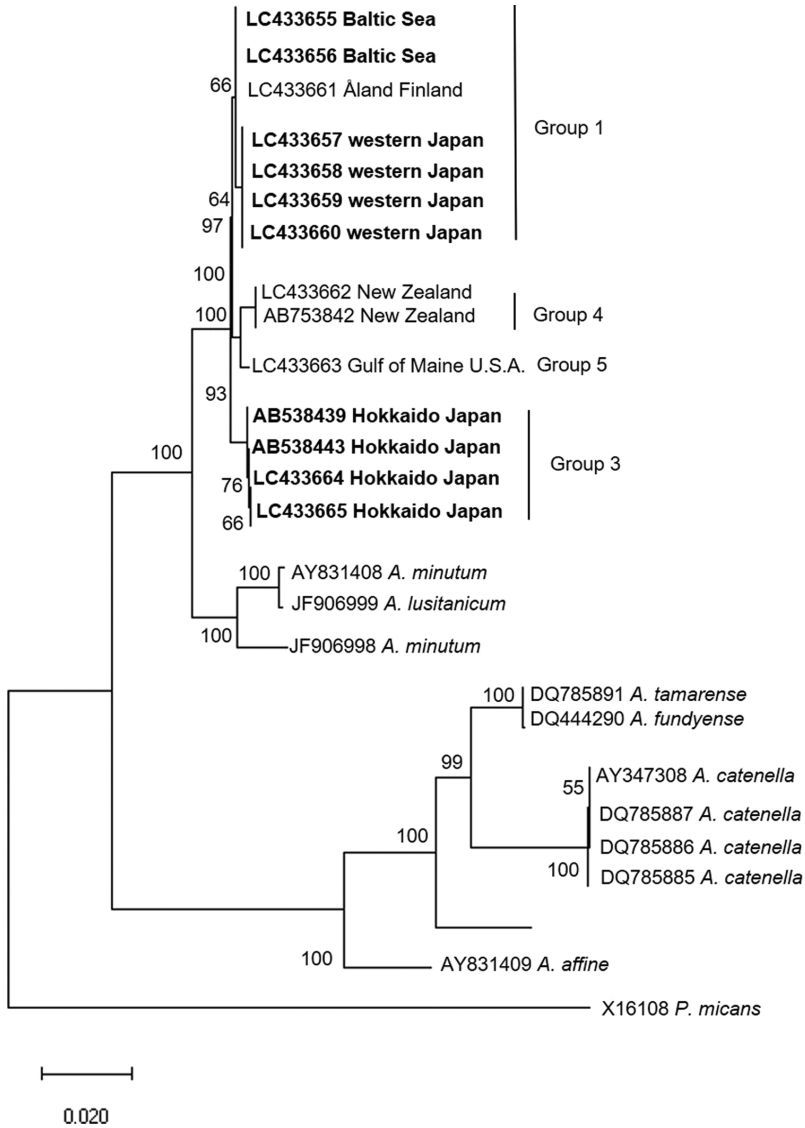

Fig. 4. A maximum-likelihood tree based on the LSU full-length alignment (3474 bp, incl. insertion/deletions) including 26 sequences and *Prorocentrum micans* serving as an outgroup. Bootstrap values > 50% are shown. Group assignments based on Kremp et al. (2014).

Tillmann et al. (2014). In case of the SSU phylogeny, the groups 1 and 2 were not clearly differentiated, group 6 was placed within the group 2 and overall the groups were not as well resolved as in the ITS and LSU phylogenies. Similar pattern for the SSU phylogeny has been previously reported by Tillmann et al. (2014) and was explained by the more conserved nature of this rDNA region compared to ITS and LSU. *Alexandrium ostenfeldii* isolates from the Baltic Sea and western Japan mainly clustered with the other isolates originating from shallow and productive coastal areas with low salinity (group 1; Kremp et al., 2014 and references therein). The isolates from northern Japan grouped separately from *A. ostenfeldii* from other locations as in previous studies (Kremp et al., 2014; Tillmann et al., 2014), where it was suggested to indicate geographical isolation (Kremp et al., 2014). This explanation is supported by the average surface current patterns between the sampling locations in western and northern Japan (Rosa et al., 2009; Ito et al., 2014) as well as by the model-simulated transport for two spring seasons, which showed a northern origin for the majority of particles transported to Funka Bay. In addition, the environmental conditions at the northern and western Japan sampling locations differ when the species is present in respective locations. For example, in Funka Bay the surface salinity is around 32 and temperature around 3° C from March to April (Shinada et al., 1999) and may increase to 6 °C by May (Azumaya et al., 2001), whereas in the Lake Koyama-ike the average salinity ranges from 4 to 9.5 and temperature 22.5–31.9 ° C during summer-autumn, potentially indicating different environmental preferences.

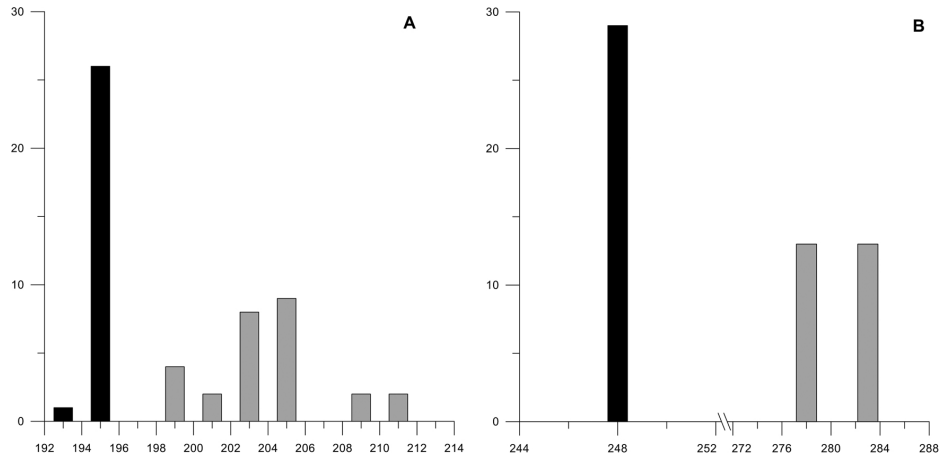

Fig. 5. Allele frequencies in loci *Aosten13* (A) and *Aosten296* (B). Y-axis shows allele frequencies and X-axis indicates specific alleles. Black bars represent allele frequencies in western Japanese and grey bars the Baltic Sea populations.

Genotyping was successful only in two out of ten microsatellite loci in western Japanese samples due to lack of PCR amplification, suggesting a mutation in the primer regions. The low average number of alleles and low genetic diversity of the western Japanese strains compared to the Baltic Sea strains might indicate a recent introduction of *A. ostenfeldii* to western Japan. The comparison of microsatellite flanking regions between western Japanese and the Baltic Sea revealed a number of differences in the sequences, and the microsatellite repeat numbers also varied, suggesting high divergence and low connectivity between the populations. Interestingly, a potential introduction of *A. ostenfeldii* from the Baltic Sea to China has been discussed due to the close genetic grouping of the Chinese strain with the strains from the Baltic Sea and the first occurrence in China in an international port located in an estuary (Gu, 2011). In phylogenetic trees based on the short ITS and LSU alignments, western Japanese strains clustered closely together with the *A. ostenfeldii* strain from the Bohai Sea, China (Gu, 2011). This pattern was replicated in the SSU phylogeny where the Chinese strain was placed within the same group with strains from the Baltic Sea and western Japan. Thus, the close grouping of western Japanese strains and the strain from the Bohai Sea might indicate a potential introduction route of *A. ostenfeldii* via China to western Japan.

As *A. ostenfeldii* has only been reported once from the Chinese coastal water based on a single cyst, this species might not be very abundant and thus the natural dispersal to western Japan over approximately 2000 km, following the annual mean surface current patterns (Lie et al., 1998; Ichikawa and Beardsley, 2002), may be unlikely. At the same time, anthropogenic introduction of *A. ostenfeldii* from China to western Japan cannot be excluded as the Chinese *A. ostenfeldii* strain originates from near the Port of Tianjin, which has a cargo exchange with the Port of Busan, South Korea, (Busan Port Authority,

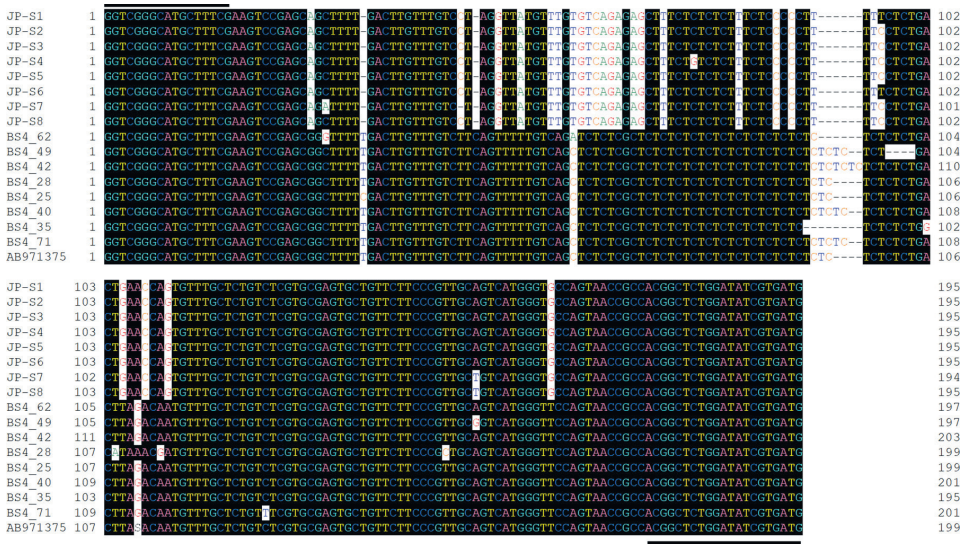

Fig. 6. Comparison between the flanking regions and microsatellite sequences from western Japan, Baltic Sea and the published sequence (AB971375) in locus *Aosten13*, horizontal bars indicate primer regions.

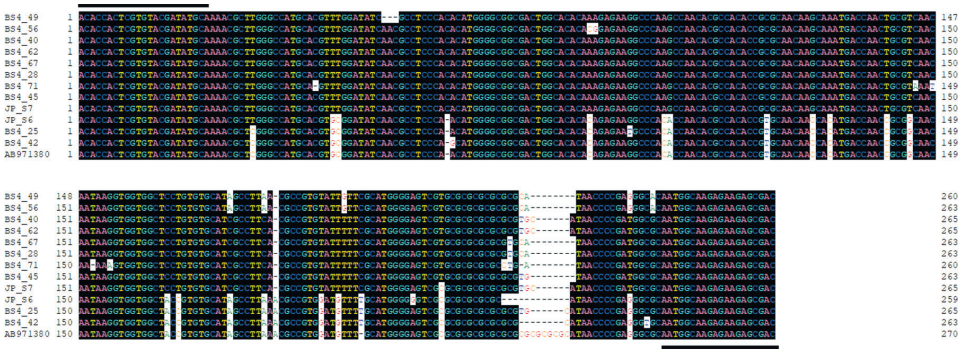

Fig. 7. Comparison between the flanking regions and microsatellite sequences from western Japan, Baltic Sea and the published sequence (AB971380) in locus *Aosten296*, horizontal bars indicate primer regions.

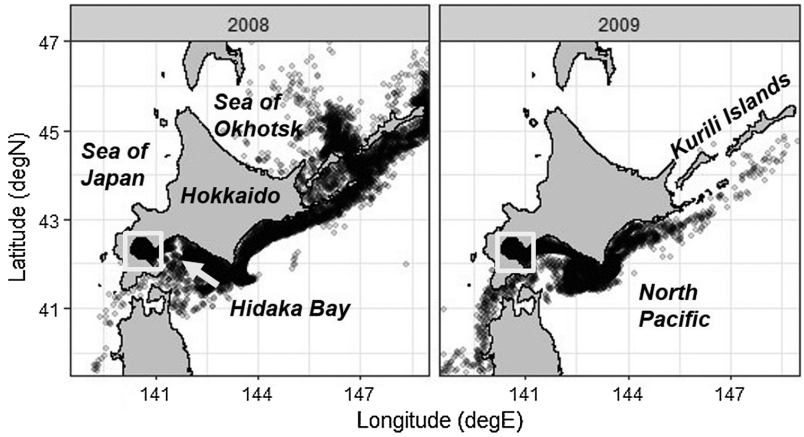

Fig. 8. Backward particle-tracking simulations from 5 to 30 m depth during 2008 and 2009 covering 60-day periods, Funka Bay is indicated by a square, an arrow indicates Hidaka Bay. The particles were released from the Funka Bay and the origin of particles is represented by dots.

2016) and gene flow between the *Margalefidinium polykrikoides* (previously *Cochlodinium polykrikoides*) populations from the coast of South Korea and western coast of the Sea of Japan has been reported (Nagai et al., 2009). Furthermore, the Port of Busan has also a cargo exchange with the Port of Kitakyushu, western Japan, (Kitakyushu Seaport and Airport Bureau, 2017) and thus the anthropogenic introduction of *A. ostenfeldii* to the west coast of Japan might be possible. Although the species has so far not been reported from the coastal waters of South Korea, the active connection between different ports provides a possibility for the anthropogenic introduction. Further transport along the western Japanese coast might be facilitated by surface currents (Lee and Niiler, 2005; Onitsuka et al., 2010) or human activities, such as transport of shellfish, as is potentially the case for *A. tamarense,* North American clade (Scholin et al. 1995) (now *A. catenella*), from the western and eastern coasts of Japan (Nagai et al., 2007; Matsuyama et al., 2008).

The first red tide of *A. ostenfeldii* in Japan appeared in Lake Koyama-ike, Tottori Prefecture in October 2013 after the opening of floodgates in March 2012. As this was the first report of *A. ostenfeldii* in the region, it is not known if the bloom was based on local seed population or instead reflects a more recent introduction. *Alexandrium ostenfeldii* produces resting cysts as a part of its life cycle (Mackenzie et al., 1996), which may provide a seed population for the bloom. Prior the bloom, salinity in the lake changed notably from < 3.25 to 14.45 (11.44 at the time of the bloom) (Nomura et al., 2009; Okamoto and Miyamoto, 2016). Thus, the bloom might have been a response of the local population, hidden flora (Smayda, 2002), to favorable environmental conditions. Bloom of a local population is not supported by the lack of previous records of *A. ostenfeldii* or other *Alexandrium* species from the lagoon (Okamoto and Miyamoto, 2016) and by limited water exchange with the Sea of Japan prior to 2012 due to the closed floodgates during vegetative growth season of *A. ostenfeldii* (spring to late autumn) (Nomura et al., 2009). Therefore, the formation of red tide after the opening of floodgates probably results from a recent introduction of A. ostenfeldii to the lagoon, where it responded to suitable environmental conditions, such as lower summer surface salinity compared to the Sea of Japan (summer salinity of > 32, (Kim et al., 2004)). Recent colonization is further supported by the low genetic diversity present in the western Japanese population. Obtaining more *A. ostenfeldii* strains from different coastal areas of Japan and neighboring countries could provide further information on the origin and genetic relatedness of different *A. ostenfeldii* populations in Japan by utilizing high-resolution genetic markers such as microsatellites or RAD/MIG-sequencing.

5. Conclusion

Phylogenetic analyses revealed a distant relationship between *A. ostenfeldii* isolates from western and northern Japan, potentially explained by the differences in habitats as well as the geographic isolation of the northern Japanese population. The isolates from western Japan

were more similar with global isolates from shallow and brackish habitats; for example the western Japanese isolates were more similar to an isolate from China than to the northern Japanese isolates. Genotyping of western Japanese isolates revealed low genetic diversity, which might be explained by a recent introduction to the area after a notable change in salinity. A further study employing more isolates from coastal areas of Japan may clarify the origin of western and northern Japanese *A. ostenfeldii* populations.

Declaration of interest

None.

Acknowledgments

A. Kondo and R. Kubota are thanked for their help with the molecular work. This study was supported by a research grant of international collaboration projected in Fisheries Research Agency of Japan (FRA) in 2010, 2014 and 2015, by a research project grant from the FRA in 2018, grants # 251564 and 310449 (A. K.), and by a grant from the Walter and André de Nottbeck Foundation (J. J.). Support for D. M. A. was provided by the National Science Foundation (OCE-1840381) and National Institutes of Health (NIEHS-1P50-ES021923-01) through the Woods Hole Center for Oceans and Human Health.[CG]

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.hal.2019.02.005.

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